



**UNIVERSITY OF  
BIRMINGHAM**

**Developing Normothermic Machine Liver Perfusion for  
Improvement of Marginal Donor Graft Quality**

**By**

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## Abstract

The studies incorporated in this thesis examined ways to develop *ex situ* normothermic machine perfusion (NMP) of the liver as a tool to enhance the reconditioning of high-risk extended criteria donor (ECD) organs. Two possible alternatives were investigated: (1) The use of hypothermic oxygenated machine perfusion as a therapeutic intervention preceding NMP; and (2) the delivery of a pharmacological combination of drugs targeting hepatic lipid metabolism during NMP. Using human donor livers discarded for transplantation, the feasibility of a combined protocol of hypothermic oxygenated perfusion (HOPE) and NMP was shown. HOPE optimised hepatic mitochondrial bioenergetic and oxidative status as well as mitigated ischaemia-reperfusion injury, while NMP maintained the organs' metabolism thus allowing the assessment of its metabolic functions. This combined protocol was facilitated with the use of a single acellular haemoglobin-based oxygen carrier (HBOC)-based perfusate throughout the entire perfusion, using a cold-to-warm machine perfusion protocol. These combined protocols enabled superior recovery of metabolic functions of ECD livers compared to NMP alone. The delivery of a combination of drugs targeting the hepatic lipid metabolism during NMP was also investigated. This approach reduced the intracellular lipid content of discarded human donor livers via enhancement of fatty-acids  $\beta$ -oxidation and solubilisation of lipids in the perfusate. The boosted lipid metabolism improved the metabolic status of the organs optimising their functional recovery and halted oxidative stress-related hepatobiliary injury. These findings are promising and guarantee future clinical investigation, opening a window of opportunity to improve the reconditioning of ECD livers.

**‘No guaranties. This is life and you should know that.’**

*From my memory, 2016*

**‘When the winds of change blow, some people build walls and others build windmills.’**

*Chinese Proverb*





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## List of Publications

### Academic Journal Papers

#### Published Manuscripts Arising from This Thesis

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**Boteon YL**, Laing RW, Schlegel A, Wallace L, Smith A, Attard J, Bhogal R, Neil D, Hubscher S, Perera T, Mirza D, Afford S, Mergental H. **Combined Hypothermic and Normothermic Machine Perfusion Improves Functional Recovery of Extended Criteria Donor Livers.** Liver Transpl. 2018;24(12):1699–1715.

**Boteon YL**, Wallace L, Boteon APCS, Mirza D, Mergental H, Bhogal R, Afford S. **An Effective Protocol for Defatting of Primary Human Hepatocytes which is Non-Toxic to Cholangiocytes or Intrahepatic Endothelial Cells.** PLoS One. 2018;13(7):e0201419.

Mergental H, Stephenson BTF, Laing RW, Kirkham AJ, Neil DAH, Wallace L, **Boteon YL**, Widmer J, Bhogal RH, Perera T, Smith A, Reynolds G, Yap C, Hubscher S, Mirza D, Afford SC. **Development of clinical criteria for functional assessment to predict primary non-function of high-risk livers using normothermic machine perfusion.** Liver Transpl. 2018;24(10):1453–1469.

Bhogal R, Weston C, Veldius S, Leuvenink HGD, Reynolds G, Davies S, Nyguet-Thin L, Alfaifi M, Shepard E, **Boteon Y**, Wallace L, Oo Y, Adams DH, Mirza DF, Mergental H, Muirhead G, Stephenson BF, Afford RB. **The ROS-Mitophagy Signalling Pathway Regulates Liver Endothelial Cell Survival during Ischaemia-Reperfusion Injury.** Liver Transpl. 2018;24(10):1437–1452.

**Boteon YL**, Boteon APCS, Attard J, Mergental H, Mirza D, Bhogal R, Afford S. *Ex situ* machine perfusion as a tool to recondition steatotic donor livers: troublesome features of fatty livers and the role of defatting therapies. A systematic review. Am J Transplant. 2018;18(10):2384–2399.

**Boteon YL**, Afford SC, Mergental H. **Pushing the Limits: Machine Preservation of the Liver as a Tool to Recondition High-Risk Grafts.** Curr Transplant Rep. 2018;5(2):113–120.

**Boteon YL**, Stephenson BTF, Neil DAH, Mirza DF, Afford SC, Mergental H. **Lipid metabolism and functional assessment of discarded human livers with steatosis undergoing 24 hours of normothermic machine perfusion.** Liver Transpl. 2018;24(5):708–709.

**Boteon YL**, Laing R, Mergental H, Reynolds GM, Mirza DF, Afford SC, Bhogal RH. **Mechanisms of autophagy activation in endothelial cell and their targeting during normothermic machine liver perfusion.** World J Gastroenterol. 2017;23(48):8443–8451.

Laing RW, Mergental H, Yap C, Kirkham A, Whilku M, Barton D, Curbishley S, **Boteon YL**, Neil DA, Hübscher SG, Perera MTPR, Muiesan P, Isaac J, Roberts KJ, Cilliers H, Afford SC, Mirza DF. **Viability testing and transplantation of marginal livers (VITTAL) using normothermic machine perfusion: study protocol for an open-label, non-randomised, prospective, single-arm trial.** BMJ Open. 2017;7(11):e017733.

Laing RW, Bhogal RH, Wallace L, **Boteon Y**, Neil DAH, Smith A, Stephenson BTF, Schlegel A, Hübscher SG, Mirza DF, Afford SC, Mergental H. **The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion.** Transplantation. 2017;101(11):2746–2756.

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Boteon APCS, **Boteon YL**, Hodson J, Osborne H, Isaac J, Marudanayagam R, Mirza DF, Muiesan P, Roberts JK, Sutcliffe RP. **Multivariable analysis of predictors of unplanned hospital readmission after pancreaticoduodenectomy: development of a validated risk score.** HPB (Oxford). 2018 Jul 23. pii: S1365-182X(18)32665-0. doi: 10.1016/j.hpb.2018.06.1802.

Piñero F, Costa P, **Boteon YL**, Duque SH, Marciano S, Anders M, Varón A, Zerega A, Poniachik J, Soza A, Padilla Machaca M, Menéndez J, Zapata R, Vilatoba M, Muñoz L, Maraschio M, Podestá LG, McCormack L, Gadano A, Boin ISFF, García P, Silva M; Latin American Liver Research, Education, Awareness Network (LALREAN). **A changing etiologic scenario in liver transplantation for hepatocellular carcinoma in a multicenter cohort study from Latin America.** Clin Res Hepatol Gastroenterol. 2018;42(5):443–452.

Boteon APCDS, **Boteon YL**, Vinuela EF, Derosas C, Mergental H, Isaac JR, Muiesan P, Mehazard H, Ma YT, Shah T, Mirza DF, Perera MTPR. **The impact of transarterial chemoembolization induced complications on outcomes after liver transplantation: A propensity-matched study.** Clin Transplant. 2018;32(5):e13255.

Piñero F, Costa P, **Boteon YL**, Duque SH, Marciano S, Anders M, Varón A, Zerega A, Poniachik J, Soza A, Machaca MP, Menéndez J, Zapata R, Vilatoba M, Muñoz L, Maraschio M, Fauda M, McCormack L, Gadano A, Boin IS, García JHP, Silva M. **Results of Liver Transplantation for Hepatocellular Carcinoma in a Multicenter Latin American Cohort Study.** Ann Hepatol. 2018;17(2):256–267.

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**Boteon YL**, Attard J, Boteon APCS, Wallace L, Reynolds G, Hubscher S, Mirza DF, Mergental H, Bhogal RH, Afford SC. **Manipulation of lipid metabolism during normothermic machine perfusion: Effect of defatting therapies on donor liver functional recovery.** Liver Transpl.

## **Submitted – Under review**

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## **Conference Presentations Arising from This Thesis**

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**Boteon YL**, Attard J, Laing R, Boteon APCS, Wallace L, Bhogal R, Reynold G, Neil D, Mergental H, Mirza D, Afford S. Pharmacological Defatting of Steatotic Human Livers Using a Novel Perfusion Solution During Normothermic Machine Perfusion. At the 2018 American Transplant Congress (ATC), American Society of Transplant Surgeons (ASTS), celebrated in Seattle, Washington, USA, on 2–6 June 2018.

**Boteon YL**, Laing R, Schlegel A, Attard J, Wallace L, Bhogal R, Neil D, Smith A, Perera T, Isaac J, Muiesan P, Mirza D, Afford S, Mergental H. Factors predicting viability achievement on discarded donor livers submitted to extra-corporeal machine perfusion. At the 2018 Congress of the International Liver Transplant Society (ILTS), celebrated in Lisbon, Portugal, on 23–26 May 2018.

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**Boteon YL**, Bhogal R, Wallace L, Laing R, Mirza D, Mergental H, Afford S (2017). The Efficacy of Pharmacological Intervention for Reducing Macrovesicular Lipid Accumulation in Primary Human Hepatocytes In Vitro: A Precursor Study to Improvement of Macrovesicular Steatosis in Donor Livers with Normothermic Machine Perfusion of the Liver. At the Liver Meeting 2017 American Association for the Study of Liver Diseases (AASLD), celebrated in Washington, DC, USA, on 20–24 October 2017.

**Boteon YL**, Laing R, Wallace L, Schlegel A, Bhogal R, Mirza D, Mergental H, Afford S. Impact of graded steatosis on the ex-vivo machine perfusion parameters of donor livers using a comprehensive protocol of perfusion techniques. At the 18th Congress of the European Society of Transplantation (ESOT), celebrated on 24–27 September 2017.

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**Boteon YL**, Laing R, Neil D, Schlegel A, Attard J, Wallace L, Reynold G, Bhogal R, Smith A, Perera T, Muiesan P, Isaac J, Mirza D, Afford S, Mergental H. Viability outcomes for donor livers discarded due to steatosis following extra-corporeal machine perfusion of the liver. At the 2018 American Transplant Congress (ATC), American Society of Transplant Surgeons (ASTS), celebrated in Seattle, Washington, USA, on 2–6 June 2018.

**Boteon YL**, Laing R, Schlegel A, Attard J, Wallace L, Smith A, Perera T, Isaac J, Mirza D, Afford S, Mergental H. Factors Predicting Viability Achievement on Discarded Donor Livers Submitted to Extra-Corporeal Machine Perfusion. At the 2018 American Transplant Congress (ATC), American Society of Transplant Surgeons (ASTS), celebrated in Seattle, Washington, USA, on 2–6 June 2018.

**Boteon YL**, Laing R, Neil D, Schlegel A, Attard J, Wallace L, Reynold G, Bhogal R, Smith A, Perera T, Muiesan P, Isaac J, Mirza D, Afford S, Mergental H. Viability outcomes for donor livers discarded due to steatosis following extra-corporeal machine perfusion of the liver. At the 2018 Congress of the International Liver Transplant Society (ILTS), celebrated in Lisbon, Portugal, on 23–26 May 2018.

**Boteon YL**, Schlegel A, Laing R, Wallace L, Smith A, Attard J, Neil D, Perera T, Mirza D, Isaac J, Muiesan P, Afford S, Mergental H. A merged protocol of hypothermic oxygenated machine perfusion and normothermic machine perfusion optimises the reconditioning of marginal human donor livers. At the International Liver Conference (ILC), European Association for the Study of Liver Diseases (EASL), celebrated in Paris, France, on 11–15 April 2018.

**Boteon YL**, Wallace L, Bhogal R, Boteon APCS, Laing R, Reynolds G, Mirza D, Mergental H, Afford S. The effectiveness of a pharmacological intervention for defatting of primary human hepatocytes and its effect on other cell types of the liver: a precursor *in vitro* study to

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**Boteon YL**, Laing R, Wallace L, Schlegel A, Boghal R, Mirza D, Mergental H, Afford S. The impact of temperature on ex-vivo machine perfusion of severely steatotic donor livers. At the 18th Congress of the European Society of Transplantation (ESOT), celebrated in Barcelona, Spain, on 24–27 September 2017.

**Boteon YL**, Laing R, Boghal R, Neil DA, Smith A, Stenphenson BTF, Schlegel A, Hubscher SG, MirzaDF, Afford SC, Mergental H (2017). The use of an haemoglobin based oxygen carrier in a human liver model of normothermic machine perfusion. At the 2017 American Transplant Congress (ATC), American Society of Transplant Surgeons (ASTS), celebrated in Chicago, Illinois, USA, on 29th April–3rd May 2017.

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## List of Abbreviations

ACOX1	Acyl-coenzyme A oxidase 1
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
BMI	Body mass index
CAR	Constitutive androstane receptor
CD	Cluster of differentiation
CIT	Cold ischaemia time
COR	Controlled oxygenated rewarming;
CPT1A	Carnitine palmitoyltransferase form 1A
DBD	Donation after brain stem death
DCD	Donation after circulatory death
D-HOPE	Dual hypothermic oxygenated machine perfusion
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DRI	Donor risk index
EAD	Early allograft dysfunction
ECD	Extended criteria donors
ET	Eurotransplant
FA	Fatty acids
GDNF	Glial cell-line derived neurotrophic factor
GGT	Gamma-glutamyl transpeptidase

GPS	Glutamine/ Penicillin/ Streptomycin
HA	Hepatic artery
HBOC	Acellular haemoglobin-based oxygen carrier
HBD	Hypoxic brain damage
HDL	High density lipoprotein
HIEC	Human intrahepatic endothelial cells
HOPE	Hypothermic oxygenated perfusion
ICH	Intracranial haemorrhage
IL	Interleukin
IQR	Interquartile range
IRI	Ischaemia-reperfusion injury
IRS	Immunoreactive score
ITBL	Ischaemic-type biliary lesions
LD	Lipid droplets
LDH	Lactate dehydrogenase
LT	Liver transplantation
MaS	Macrovesicular steatosis
MELD	Model for end-stage liver disease
MiS	Microvesicular steatosis
MP	<i>Ex situ</i> machine perfusion of the liver
MRCP	Magnetic resonance cholangiopancreatography
NMP	<i>Ex situ</i> normothermic machine perfusion
NAFLD	Non-alcoholic fatty liver disease
NAS	Non-anastomotic stricture
NR	Nuclear receptors

NRP	<i>In situ</i> normothermic regional perfusion
8-OHdG	8-hydroxy 2 deoxyguanosine
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
PHH	Primary human hepatocytes
PNF	Primary non-function
PPAR	Peroxisome proliferator-activated receptor
PKA	Protein kinase A
PV	Portal vein
PXR	Pregnane X receptor
RBC	Red blood cells
ROS	Reactive oxygen species
SCS	Static cold storage
SMP	Subnormothermic machine perfusion
TG	Triglycerides
TNF- $\alpha$	Tumor necrosis factor $\alpha$
UCP2	Uncoupling protein 2
UK	United Kingdom
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very-low density lipoprotein
WIT	Warm ischaemic time
4-HNE	4-hydroxynonenal

## **Chapter 1**

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**GENERAL INTRODUCTION: THE CURRENT CHANGE IN  
DONOR CHARACTERISTICS AND THE NEED TO INCREASE  
UTILISATION OF EXTENDED CRITERIA DONOR ORGANS.  
WHAT IS THE ROLE OF MACHINE PERFUSION OF THE  
LIVER?**

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## **1.1 Historical perspective of liver transplantation**

The first clinical series of liver transplantations (LT) was described by Starzl *et al.* in 1963 (1). Of the three reported cases, the first died during the operation due to bleeding and the other two lived for 22 and 7 days, respectively. In 1968, Starzl *et al.* reported another seven cases of LT: Four of the patients died between two and six months post-procedure, while the other three remained alive, one of whom completed a 12-month follow-up post-transplantation at the time of the report (2). Initial technical challenges of the procedure, such as preservation of the donor liver and surgical technique, have been discussed by Roy Calne and Roger Williams when reporting five cases in the United Kingdom (UK) (3).

Further advances were achieved with the definition of irreversible coma as a criterion for death in 1968 (4), and advances in immunosuppressive regimen in following decades. Cyclosporine was introduced in the seventies, and revolutionised post-transplant survival rates (5). In 1989, 5-year survival rates post-procedure reached 64% with the use of cyclosporine (6). In the late eighties and early nineties, tacrolimus was introduced by Starzl (7, 8). Tacrolimus was shown to be more effective than cyclosporine in reducing the occurrence of acute cellular rejection, as well as improving graft and patient survival after the procedure. Thus, the use of tacrolimus has become the gold standard immunosuppressive regime (9).

Since the nineties, LT has spread to many parts of the world. It is the only definitive therapeutic option for end-stage liver diseases and a curative treatment for early-stage hepatocellular carcinoma (10). The currently 1-year and 5-year patient survival rates in the UK for donation after brainstem death (DBD) LT are as high as 93% and 81%, respectively (11). In the United States of America (USA), 5-year survival rates are 81.2% (12).

However, LT has become a victim of its own success. With an ever-growing number of patients on waiting lists for LT, the supply of donor organs has largely exceeded the demand and many countries have been observing high waiting-list mortality rates (13). It is estimated that over the past decade, the number of patients on the transplant waiting list in the UK has almost doubled (14). This continuously increasing demand for LT is contemporarily driven mainly by a global climbing prevalence of non-alcoholic fatty liver disease (NAFLD). Indeed, NAFLD is one of the leading reasons for LT (15). It is considered the hepatic manifestation of metabolic syndrome and its compounds, such as obesity, diabetes and hypertension (15).

## **1.2 Change in donor characteristics and the Extended Criteria Donors**

In the UK, between April 2017 and March 2018, the total number of organ donors increased approximately 10%, while the number of patients on the LT waiting list decreased by 32% (11). Despite these advances in absolute numbers, there is still a discrepancy between the availability of donor organs for transplantation and patients on the waiting list. Consequently, 11% of patients listed for LT between 2015 and 2016 died while waiting for an organ for the procedure (11).

This discrepancy is mainly associated with a significant change in donor characteristics. The Transplant Activity Report 2017-2018, from the National Health Service Blood and Transplant (NHSBT), states that donors are becoming older, more obese and are more frequently dying from non-traumatic causes (11). This report indicates that, between 2008 and 2018, there was an 8% increase (20–28%) in the proportion of donors with a body mass index (BMI)  $> 30 \text{ kg/m}^2$ , and a 9% (5–14%) increase in donors older than 69 years (11). These changes negatively impact the quality of donor organs. Therefore, the percentage of donor livers discarded for transplantation after retrieval has consequently increased from 12% to 15% over the same

period. Stratifying this discard rate by donor type, between April 2017 and March 2018, the discard rate reached 9% for donation after brain stem death (DBD) and 33% for donation after circulatory death (DCD). Moreover, clinical unsuitability was the most common reason for discard donor organs. Clinical unsuitability was defined in the document as poor perfusion, prolonged preservation times and donor history (11). In a previous and more detailed version of the report (2016), steatosis was the most frequent reason within this wide category of clinical unsuitability (16). In the USA, the last annual report on LT noted a discard rate of 9% for donor livers following retrieval (12). However, these rates were higher for older donors and DCD organs (12).

In the UK, 26% of deceased donor livers transplanted between April 2017 and March 2018 were from DCD donors (11). Donation after circulatory death transplantation is associated with high-rates of ischaemic-type biliary lesions (ITBL) and shorter patient and graft survival (17–19). Donor organs with steatosis, organs that have been exposed to prolonged preservation times or organs from elderly donors are all more vulnerable to ischaemia-reperfusion injury (IRI), and therefore, are referred to as marginal or extended criteria donor (ECD) organs (20). However, the defining parameters of ECD are not definitive, and can vary slightly between centres (21), although donor age greater than 60, steatosis rates more than 15% and cold ischaemia times longer than 10 hours are frequently quoted as possible thresholds (22). Moreover, ECD liver transplantation is associated with high rates of primary non-function (PNF) and/or delayed graft function (DGF), as well as higher morbidity and mortality rates (23–25). In a recent publication, which reported on the outcomes of 611 LT recipients, age > 65 years, macrovesicular steatosis on biopsy > 40% and cold ischaemia time > 14 hours were identified as major risk factors of inferior patient and graft survival (26). Despite these unfavourable outcomes, the utilisation of ECD livers is necessary due to contemporary changes

in donor characteristics and the ever-growing discrepancy between supply and demand for donor organs.

### **1.3 Strategies to improve utilisation of Extended Criteria Donor Livers**

#### **1.3.1 On the recipient side**

To increase utilisation rates of ECD livers, many centres have begun allocating these high-risk organs to less sick patients on the waiting list (25, 27, 28). Whilst donor risk factors associated with inferior outcomes post-transplantation have previously been described, their interaction with recipient characteristics has not yet been investigated in-depth. As a result, researchers have begun to develop clinical prognostic models in an attempt to balance donor and recipient risks. The D-MELD, the product of donor age versus the Model for End-Stage Liver Disease (MELD) score of the recipient, was created as an attempt to enable a safer donor/recipient match (29). A result greater than 1,600 is associated with poor post-transplant outcomes, such as shorter patient survival and prolonged length of stay (29). Later, the Zurich group developed the Balance of Risk (BAR) score (30) utilising The United Network for Organ Sharing (UNOS) database (37,255 patients). In their study, six risk factors were associated with inferior post-operative outcomes, namely the recipient's MELD score, cold ischaemia time, recipient age, donor age, re-transplantation, and life support dependence prior to LT (30). The authors concluded calculating the BAR score optimises the recipient's survival/resource utilisation ratio.

More recently, a new predictive clinical score was developed for DCD livers specifically. The UK DCD Risk Score aims to assess the risk of DCD LT based on donor and recipients features (31). A large cohort of 1,153 recipients was used to develop the score, which was then validated using the UNOS database. The authors identified the following seven independent predictors

of poor graft survival: functional donor warm ischaemia time, cold ischaemia time, recipient MELD score, recipient age, donor age, previous LT and donor BMI. A score greater than 10 predicted, with good accuracy, graft loss for PNF and ITBL (31). Interestingly, this risk score not only predicts the risk of donor/recipient matchings, but also determines whether an organ requires optimisation before transplantation through the machine perfusion of donor livers.

Whereas risk-assessment predictive tools are important to optimise donor/recipient matching, the capacity of the sickest recipients to wait for a standard donor organ should be taken into consideration. For example, Jennifer Lai (2017) proposed an interesting framework that considers pre-transplant patient vulnerability in the decision-making process of accepting an ECD liver (32). Transplantation of a high-risk ECD liver into a highly-vulnerable patient would make transplantation futile. This is due to the fact that the patient, most likely, does not have enough metabolic reserve to cope with a complicated postoperative period (32).

Altogether, these proposals reveal the complexity of optimising ECD liver utilisation. In addition, the transplant community should be careful to not penalise less sick patients by transplanting them with high-risk ECD organs. Therefore, to solve this conundrum, strategies to alleviate the risks associated with ECD LT are required. One option is to consider better techniques of organ preservation, thereby preventing further damage to such highly vulnerable organs. Moreover, the possibility of reconditioning the organs prior to LT should be considered.

### **1.3.2 On the donor organ side**

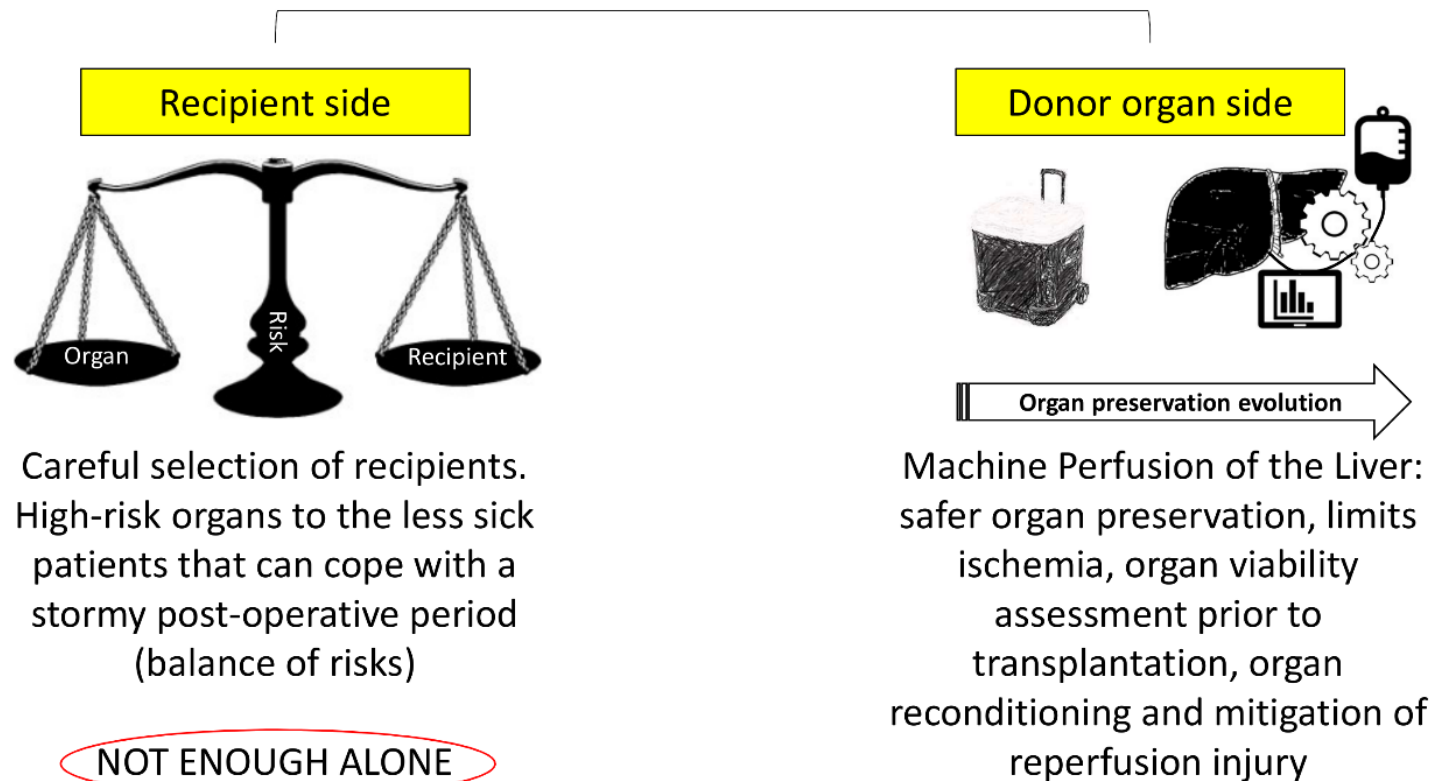
Current changes in donor characteristics, as previously described, are likely to persist over time, or even worsen with an upward trend of elderly and obese donors. Whilst transplant surgeons do not have control over these donor features, they can consider alternatives to better preserve or even recondition ECD livers. Traditionally, donor livers are stored on ice and immersed in

preservation solutions after retrieval. This method has proven to be highly effective at successfully preserving donor organs thus far (33). Nevertheless, the wider utilisation of ECD livers has exceeded the preservation capacities of traditional static cold storage (SCS). Consequently, as mentioned previously, ECD LT using SCS is associated with inferior outcomes (23, 24, 26, 34).

Attempting to overcome the risks associated with ECD LT, the transplant community has thus far attempted to minimise recipient risk factors. This strategy centres around a more appropriate donor/recipient matching. On the donor organ side specifically, transplant surgeons balance donor risk factors when making utilisation decisions about poor-quality organs. For example, combinations of steatosis and DCD status, or advanced age and DCD status, are avoided when considering the transplantability of an organ (35). Consequently, 17% of donor livers retrieved in the UK are not transplanted (11). While these organs are being discarded, patients are dying on the waiting list or their condition is worsening to the point that transplantation is no longer a viable option. Therefore, new strategies for organ preservation, or even organ reconditioning, are urgently needed. Figure 1.1 presents a diagrammatic summary of strategies to increase utilisation of ECD livers.

Figure 1.1: Current strategies available to increase safely utilisation of extended criteria donor organs.

## How to minimise the risks associated with extended criteria donors in liver transplantation?



### 1.3.2.1 Machine perfusion of the liver

Lindbergh and Carrel (1935) first reported the use of an *ex situ* device to sustain prolonged organ functioning via perfusion with oxygenated serum at normothermia (36). While this concept of dynamic organ preservation was investigated later in canine models by Starzl *et al.* (37), the development of effective cold preservation solutions dampened interest in this approach. Since cold preservation solutions allowed for the safe static storage of organs, thus facilitating the process of organ preservation and transportation, SCS became the gold standard for organ preservation and dynamic techniques, such as the device recommended by Lindbergh and Carrel, were abandoned (38–40).

Dynamic preservation techniques resurged, however, due to the increase in utilisation of high-risk livers, such as ECD and DCD organs. Due to their high vulnerability to ischaemia and poor post-transplant outcomes following SCS, machine perfusion (MP) of the liver is considered to be a possible alternative preservation method. The use of this device may offer several advantages compared to SCS, including superior organ preservation limiting ischaemia, assessment of organ function before transplantation and the possibility to improve or repair these highly vulnerable organs. Nevertheless, benefits may vary between different modalities of MP depending on the characteristics of the perfusion; for example, timing, temperature, level of oxygenation, and constitution of the perfusate.

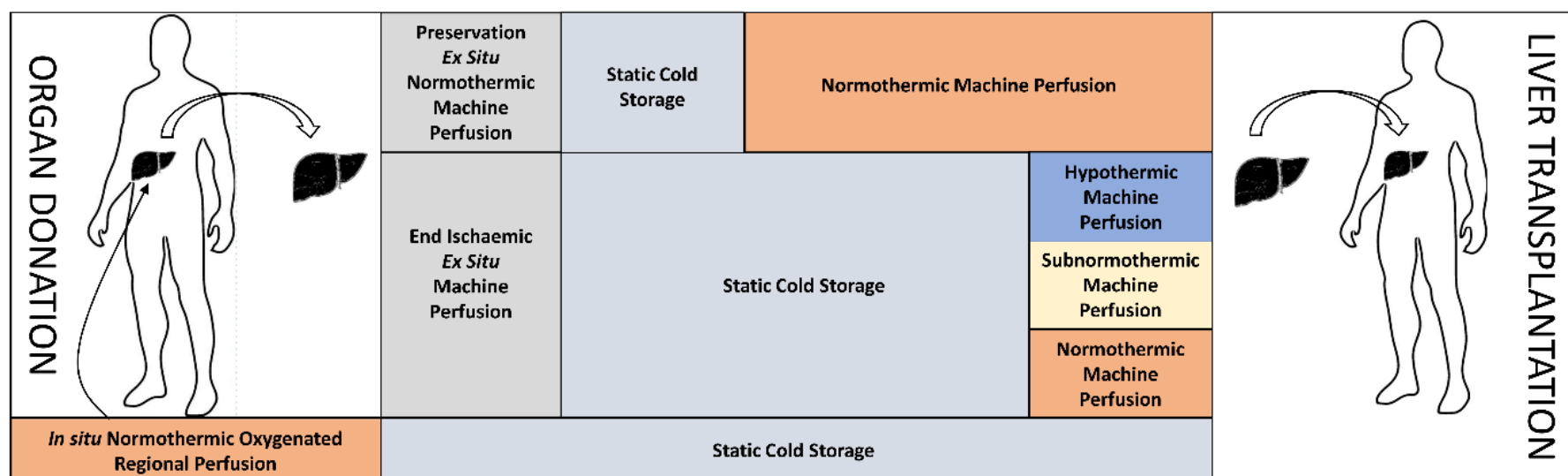
First, it is worth noting that an MP can be performed *in situ* during donor organ procurement, as well as *ex situ* after the organs have been harvested. Second, the temperature of the perfusate pumped through the liver can vary from hypothermic temperatures (10 °C) to normothermic (37 °C). In addition, the perfusate has been actively oxygenated in some studies, but not in others. Finally, regarding the timing for the use of the device, MP can be performed: (1) as a



preservation method, fully replacing SCS during organ transportation from the retrieval to the transplant centre; or (2) in the end-ischaemic method, wherein the organ is transported to the transplant centre in SCS and then undergo MP before transplantation. Figure 1.2 represents the possible timings for performing MP of the liver, from organ donation to liver transplantation.

The most studied modalities of MP of the liver will hereafter be discussed. Technical aspects and overall comments about each technique are initially presented, and then their benefits/limitations are outlined.

**Figure 1.2: Possible timings for performing machine perfusion of the liver from the organ donation until transplantation and possible techniques available for each approach.**



#### 1.3.2.1.1 *In situ* machine perfusion of the liver

This modality of MP is performed during the organ retrieval operation, or even before, via cannulation of the aorta/iliac arteries or cava/iliac veins. A balloon, or a clamp, is positioned on the descending thoracic aorta to prevent perfusion of the heart and brain. The donor blood, or a preservation solution, is used as a perfusion fluid. Thus far, *in situ* liver perfusion has only been performed at 37 °C and is therefore known as oxygenated normothermic regional perfusion (NRP) (41). Normothermic regional perfusion is considered crucial for the recovery of uncontrolled DCD organs (witnessed cardiac arrest without response to resuscitative measures), since donors would otherwise experience a prohibitively long warm ischaemic time, from asystole to the cannulation of the vessels and could flush (42). Furthermore, NRP is also considered to be beneficial within the context of controlled DCD (withdrawal of life support in patients with irreversible clinical conditions), when the warm ischaemic time is carefully assessed (43).

**Benefits:** Normothermic regional perfusion re-establishes the delivery of oxygen to the organs following asystole in the donor and thus, limits the injury associated with a longer warm ischaemia period (42, 43). Full metabolism recovery of the organs allows for an assessment of the markers of cellular injury and/or function prior even to SCS (41). Assessment of organ function is the cornerstone of safe utilisation of ECD organs. Until now, NRP studies have evaluated the release of transaminases in the perfusate, macroscopic appearance of the organ, bile production and some other parameters of the blood-gas analysis (such as lactate clearance, base excess and bicarbonate levels), together with donor characteristics, to predict the function of a liver post-transplantation (44). In addition, the presence of flow through the vessels facilitates organ retrieval and may deter organ injury (44).

**Limitations:** No published study has performed yet an in-depth analysis of the mechanistic effect of the procedure on the metabolism of donor organs. Thus, further investigation is needed in terms of the impact of NRP on mitochondrial injury and function, oxidative injury and downstream activation of the inflammatory cascade. Additionally, the criteria for transplantability of the organs are not clear, and many organs are refused during perfusion (45). Therefore, it is necessary to determine whether this technique can improve the organs, or if the beneficial effects seen are just the result of organ selection.

#### **1.3.2.1.2 *Ex situ* machine perfusion of the liver**

The majority of MP protocols are performed once organ retrieval is finished, therefore, are classified as *ex situ* MP. These protocols may vary in terms of temperature and extent of oxygenation of the perfusion fluid. The details of the different protocols, as well as the benefits and limitations are discussed in this section.

##### **1.3.2.1.2.1 Hypothermic machine perfusion of the liver**

For hypothermic machine perfusion (HMP), the temperature of the perfusate is generally around 10 °C (46–50). Guarrera *et al.* (2010) first published a series of 20 cases of standard DBD livers transplanted following a period of HMP (46). The perfusion was done via hepatic artery and portal vein without active oxygenation of the perfusate in an end-ischaemic model. Postoperatively, the recipients of the perfused livers experienced less early graft dysfunction and lower levels of transaminases within the first seven postoperative days, as well as a shorter hospital stay compared to the recipients of non-perfused donor organs (46). More recently, the authors applied the same protocol to high-risk DBD livers and reported lower rates of biliary complications post-transplantation (47). Subsequently, the Zurich group developed the hypothermic oxygenated perfusion (HOPE) technique, which differs from the previous

technique in that the perfusate is actively oxygenated and perfusion solely occur via the portal vein. Clinical studies have only employed HOPE to investigate DCD organs thus far, and they have confirmed the positive impact of HMP on intrahepatic biliary complications (48, 49). The Groningen group has been working on dual vessel (hepatic artery and portal vein) hypothermic oxygenated perfusion (D-HOPE) (51). In 2017, the first clinical study to use D-HOPE for 10 DCD livers transplanted found a reduction in intrahepatic biliary complications (50).

The majority of mechanistic studies on HMP that have been published thus far were undertaken by the HOPE group (52–54). During ischaemia, succinate will accumulate in the mitochondria, as in the absence of oxygen, succinate will function as an acceptor of electrons in the electron transport chain (ETC). During reperfusion, the metabolism of the accumulated succinate momentarily blocks the ETC, causing the reverse flow of electrons, which will then leak and result in the production of reactive oxygen species (ROS). Exaggerated ROS production causes oxidative tissue injury and activation of the inflammatory cascade, which in turn perpetuates tissue injury. It has been proposed that the delivery of oxygen at hypothermic temperatures leads to consumption of the accumulated succinate, enhancing mitochondrial oxidative function prior to reperfusion (55). This hypothesis is sustained by experimental studies that have found a decrease in the expression of markers indicating oxidative tissue damage, and an activation of Kupffer cells and leukocytes; In addition, these studies have found a lower release in the perfusate of markers of mitochondrial injury, damage-associated molecular pattern (DAMPs) and cytokines in livers after reperfusion following the HOPE procedure (22, 54, 55). These changes occur concomitantly with signs of down-regulation in mitochondrial respiration and enhanced ATP synthesis (22, 54, 56).

**Benefits:** Patients enrolled in clinical studies employing HMP, mainly the oxygenated modalities (HOPE and D-HOPE), exhibit significantly lower rates of intrahepatic biliary

complication post-transplantation (48–50). Moreover, HMP does not require the use of oxygen carriers in the perfusate, since the oxygen supplied to the cells through diffusion is adequate to supply the diminished metabolic demand of the organ at a low temperature. Failure of the perfusion device can be safely managed by returning the organ to SCS without risk to the organ.

**Limitations:** The diminished metabolic rate of the organs at 10 °C limits their functional assessment during HMP. One promising approach that may guarantee future studies is the investigation of dynamic changes in the mitochondrial bioenergetic status. Without any readily available parameter thus far, the threshold to stop HMP or the response of individual organs to the treatment is not so clear. The minimum perfusion period recommended is one hour, as it is advocated that 1–2 hours of oxygenated HMP is enough to replenish the ATP content of the mitochondria (22). However, despite a general orientation, a definition of the biomarkers to individualise perfusion times and assess responses to treatment in real-time is still pending. This real-time assessment of the effects of HMP on the organs is highly relevant in a context where a previous experimental model using intravital microscopy has shown that prolonged HMP may impair parenchymal perfusion (57).

#### **1.3.2.1.2.2 Subnormothermic machine perfusion of the liver**

Subnormothermic machine perfusion (SMP) is performed usually at around 20 °C with active oxygenation of the perfusion fluid via the portal vein and hepatic artery. The requirement of an oxygen carrier in the perfusate at this temperature, however, is unclear and it has not been always used (58–60). Defenders of this technique advocate that the increase in the organ's metabolic rate, as a result of the increase in temperature (from 10 °C to 20 °C), is sufficient for viability testing (58). In addition, it has been suggested in transplant animal models that SMP can positively impact mitochondrial function, increase organs' ATP stores, decrease the release

of markers of tissue injury (such as transaminases and cytokines) and improve graft function postoperatively (58, 60).

Despite the promising conjectures, no clinical studies have yet transplanted human livers following SMP. Bruinsma *et al.* (2014) performed SMP on seven discarded donor human livers; Interestingly, the authors have found that the organs metabolised lactate effectively, increased ATP stores along the perfusion and exhibited other parameters of appropriate liver metabolism (evidence of urea, albumin and bile production) without evidence of tissue injury, as assessed histologically and by the lower release of ALT and lactate dehydrogenase (LDH) in the perfusate (61). This study reinforces the hypothesis that it is feasible to assess the metabolism of human livers during SMP.

Minor *et al.* (2013) proposed a variant of the SMP technique, the controlled oxygenated rewarming (COR) (62). This method involves dual perfusion (hepatic artery and portal vein) of organs in a highly oxygenated perfusion fluid without any oxygen carrier. Total perfusion time is 90 minutes; The perfusion starts at 8 °C, and gradually increases to 20 °C within 60 minutes. In a reperfusion porcine model using *ex situ* NMP, compared to HMP or SMP alone, COR was found to increase cellular ATP stores and decrease the release of lipid peroxides and markers of hepatocellular injury (AST and ALT) in the perfusate after reperfusion. During NMP, organs that had undergone COR exhibited increased bile production, lower vascular resistance and decreased expression of proinflammatory genes (intercellular adhesion molecule 1, toll-like receptor 4 and tumour necrosis factor alpha) (62). In 2016, a clinical series of 6 DBD livers transplanted following the application of COR reported a 50% reduction in peak serum AST postoperatively, compared to SCS preserved livers, with 100% 6-month patient and graft survival (63).

**Benefits:** In a study on discarded human livers, SMP was shown to be able to sustain liver metabolic rates to a high enough level to enable viability assessment; SMP also enhanced ATP production without any evidence of tissue injury during the procedure (61). The COR protocol was suggested to yield even better results than SMP alone on an experimental model. Moreover, it was shown to be safe and significantly decrease the release of tissue injury markers after transplantation (62, 63).

**Limitations:** Only experimental studies have been published on SMP so far (60, 61), thus limiting further consideration for this technique, since the final testing for safety and effectiveness in this context is transplantation. While it has been suggested that COR can better preserve donor organs, the studies reported minimal changes in the metabolic parameters, such as bile production and lactate metabolism, of the organs during perfusion (62, 63). Consequently, COR may prevent the possibility of a functional assessment of an organ achieved during SMP.

#### **1.3.2.1.2.3 Normothermic machine perfusion of the liver**

*Ex situ* normothermic machine perfusion (NMP) is performed at 37 °C, and requires active oxygenation of the perfusate and cannulation of both the hepatic artery and portal vein. At a physiological temperature, the organs will be fully functional, allowing for an assessment of their metabolic parameters prior to transplantation (64, 65). This is vital to safely increase the utilisation of ECD livers, even if the ideal criteria of organ viability are still under investigation (64).

According to the Birmingham viability criteria, lactate clearance to levels of less than 2.5 mmol/L within 4 hours is a major criterion for considering an organ transplantable. This should be accompanied by two of the minor criteria: perfusate pH > 7.3, evidence of bile production,



metabolism of glucose, homogeneous perfusion with a soft parenchyma, arterial flow > 150 mL/min and portal vein flow > 500 mL/min. These criteria were validated in a clinical study wherein 5 discarded donor livers were successfully transplanted following an objective assessment of organ function during NMP (65). In addition, they were used in the Viability Testing and Transplantation of Marginal Livers (VITTAL) study (ClinicalTrials.gov Identifier: NCT02740608), which finished recruitment in early 2018 and are still awaiting results (66). More recently, Watson *et al.*, in a clinical series of 22 livers transplanted following NMP, proposed a comprehensive viability criteria that take into consideration markers of hepatocellular metabolism (as assessed by peak lactate fall  $\geq 4.4$  mmol/L/Kg/h; stable perfusate pH > 7.2 without sodium bicarbonate supplementation greater than 30 mmol; falling glucose beyond 2 hours, or < 10 mmol falling after 2.5 g challenge), hepatocellular injury (Alanine aminotransferase [ALT] perfusate levels < 6,000 UI at 2 hours of perfusion) and biliary function/injury (bile pH > 7.5; bile glucose < 3 mmol/L or > 10 mmol/L less than the perfusate) (67).

After a period of SCS, NMP was performed in the two clinical series described above using an end-ischaemic approach. In this method, the organs are cold flushed *in situ*, preserved in SCS during the transport to the transplant centre as routine, and then placed on an NMP. Whilst end-ischaemic NMP facilitates logistical aspects of the perfusion, such as avoiding device and perfusion team mobilisation to the retrieval centre, it does not prevent injury associated with SCS. Alternatively, NMP can be used in a preservation approach, fully replacing SCS. In this method, after the donor retrieval operation finishes, the organ is prepared for transplantation by being placed in a basin of cold fluid and smashed ice, following standard protocol (68). The common hepatic artery, portal vein, vena cava and common hepatic duct are cannulated, and the perfusion begins. The donor organ will then be transported on the device and remain there

until transplantation (69). Nasralla *et al.* (70) recently published a clinical trial reporting the results of transplantation of 121 donor livers following preservation NMP. The authors found a 50% decrease in the release of aspartate transaminase (AST) in the recipient within the first 7 postoperative days in comparison with grafts that had SCS (70).

**Benefits:** Independent of the NMP approach used, assessment of organ functioning prior to transplantation is the cornerstone of NMP utilisation. Identification of ECD livers at a high risk of graft dysfunction postoperatively may prevent recipient morbidity and mortality. However, the benefits of having an organ at full metabolism are not limited to viability assessment. Indeed, NMP allows for the administration of pharmacological therapies to donor organs (71). Such therapies may include agents that target the mitigation of IRI or the provision of drugs to accelerate the intracellular lipid metabolism leading to steatosis reversal or defatting (72). Another potential advantage of NMP is the possibility of prolonged organ preservation, which may help improve logistics in busy transplant centres (70, 73).

**Limitations:** Recovery of the organ's full metabolism imposes some requirements on this technique. First and foremost, it requires the use of an oxygen carrier in the perfusate to deliver adequate oxygen levels to supply the high metabolic demand of the organs. Human red blood cells have been traditionally used for this purpose. However, human red blood cells are a precious resource with limited availability, which also carries a low, but not insignificant risk of microbiological contamination of the organ as well as requires well-controlled storage conditions with a relatively short shelf half-life (60). For this reason, acellular haemoglobin-based oxygen carrier (HBOC)-based perfusate have been investigated as an alternative to replace human red blood cells during NMP (74). Moreover, failures to identify problems with the cannulation of vessels may induce a new period of warm ischaemia, which can ultimately result in the loss of the donor organ (75). In addition, experimental models have shown that

even without the presence of leukocytes and platelets in the circuit, NMP mimics IRI to some extent, causing oxidative tissue injury and activation of the inflammatory cascade (54, 55).

#### **1.4 Thesis plan and aims**

Despite hypothetical advantages of preservation NMP over an end-ischaemic approach, as it shortens the ischaemic injury to the organs, published studies have not yet proven its superiority (51). End-ischaemic NMP facilitates the logistics of organ perfusion while avoiding the mobilisation of the perfusion team and the device, and it has the potential to assess organ function prior to transplantation (64, 65, 67). Despite ongoing discussions about the need for more appropriate viability criteria during NMP (64), the Birmingham criteria have been shown to be in a pilot clinical series, at minimum, safe for assessing the transplantability of discarded marginal livers (65). However, approximately 40% of the high-risk ECD donor livers undergoing end-ischaemic NMP are deemed non-viable according to these criteria. This data may suggest that: (1) In the interest of patient safety, the criteria are highly selective; or (2) While NMP, which re-establishes the organ's full metabolism, allows for a viability assessment, its 'resuscitative' capacity is potentially limited. Therefore, an additional therapeutic intervention may be required.

While patient safety is central, additional therapeutic interventions during NMP may enhance organ reconditioning. In accordance, this thesis investigates opportunities for the further development of NMP to improve donor organ quality.

The aims of this thesis are:

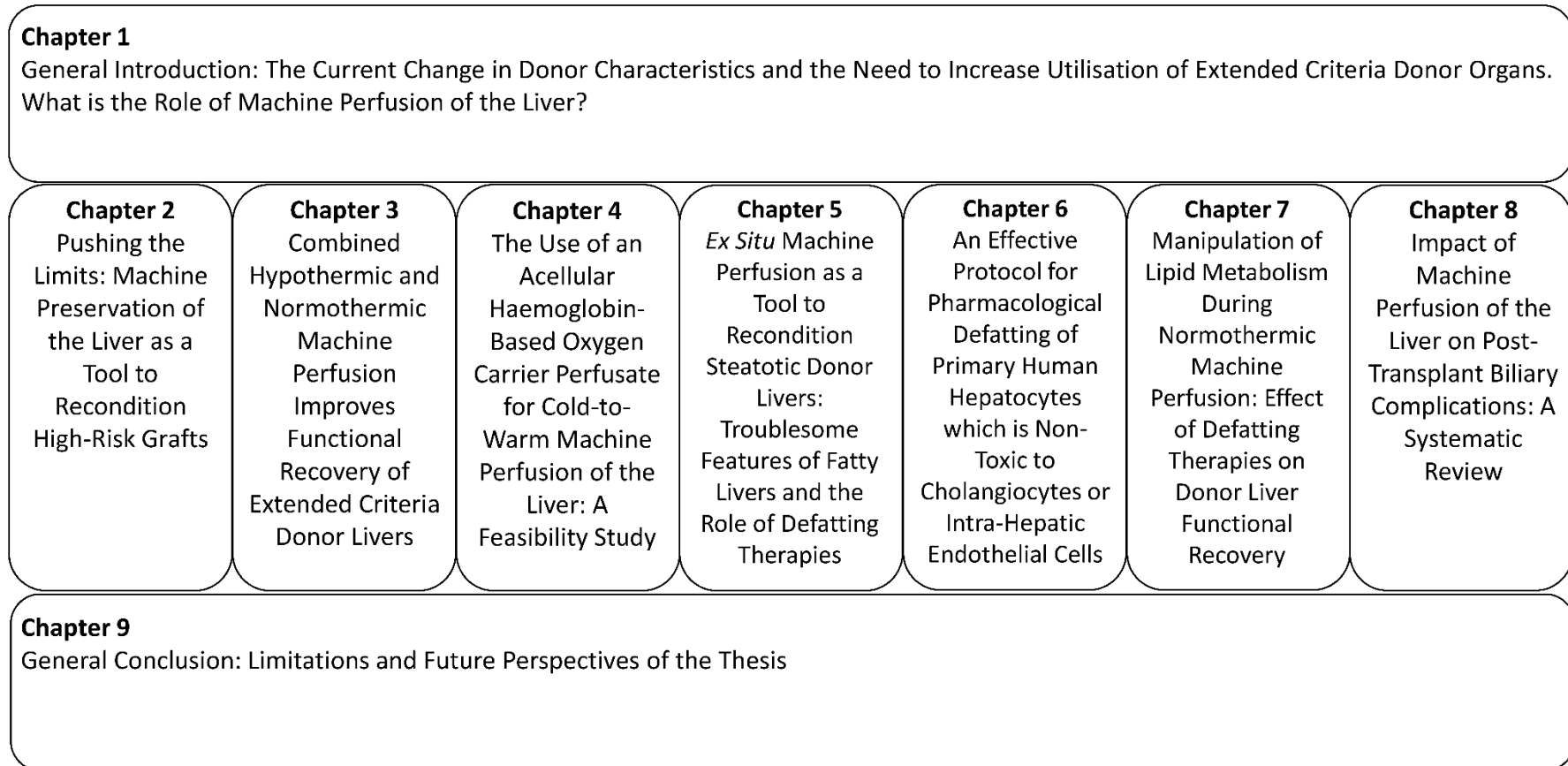
- To investigate whether a combined protocol of *ex situ* hypothermic and normothermic perfusions of the liver derive the benefits of each individual technique and, more

importantly, whether this combined protocol positively impacts the reconditioning of discarded human donor organs.

- To test, *in vitro*, the cytotoxicity and effectiveness of a combination of defatting drugs on the primary human cells of the liver, including primary human hepatocytes, intrahepatic endothelial cells and cholangiocytes.
- To investigate the feasibility of the delivery of this combination of defatting drugs to steatotic human donor livers undergoing end-ischaemic normothermic machine perfusion; To also determine whether this intervention: (1) promotes the mobilization and metabolization of intracellular lipid stores leading to defatting; (2) positively impacts the recovery of the organs' metabolic activity.

The study framework is presented in Figure 1.3. A brief summary of the studies undertaken for the development of this thesis will follow hereafter.

**Figure 1.3: Thesis overview with chapters title.**



**Chapter 2:** This chapter reviews the clinical studies on MP of the liver published so far, and discusses how MP can be a tool to recondition high-risk grafts. After an initial overview of MP in Chapter 1, Chapter 2 describes where we stand with clinical studies. Vital targets of MP to increase the utilisation of high-risk organs are discussed, including promising future interventions.

**Chapter 3:** This chapter presents the results of a newly developed protocol of MP that combines HOPE and NMP. Whilst individual characteristics of each MP technique (including temperature, levels of oxygenation, requirement of an oxygen carrier in the perfusate) result in the specific benefits associated with that protocol, they also impose limitations. For example, although HOPE has been shown to enhance the bioenergetic status and oxidative function of organs, the lower metabolic rate limits the viability assessment of the organs thus far. Conversely, NMP allows for an assessment of the metabolic functions of an organ at full metabolism, though it inevitably results in reperfusion injury to some extent. Combining these protocols, seen as divergent so far, was shown to derive individual benefits that improve the functional recovery of ECD livers.

**Chapter 4:** Following Chapter 3, which suggests that a combined protocol of hypothermic and normothermic perfusions is superior to NMP alone in the reconditioning of high-risk ECD livers, Chapter 4 proposes a refinement of the combined MP protocol. The requirement for an oxygen carrier during NMP, and the impossibility of using red blood cells during HMP, made the exchange of perfusates in the combined perfusion mandatory. To prevent entirely any concerns of exposing the organs to additional unnecessary ischemia time to switch the

perfusate, Chapter 4 investigates the feasibility of using an acellular haemoglobin-based oxygen carrier (HBOC)-based perfusate during HMP, and then rewarming it until normothermia. This approach allows for an uninterrupted combination of HMP and NMP, the Cold-to-Warm MP of the liver, which may increase its clinical applicability.

**Chapter 5:** This chapter initially discusses fatty liver disease within the context of organ donation and transplantation. Steatosis is one of the leading reasons to discard donor organs globally due to their inferior postoperative outcomes, as reported in clinical series. Following the introduction to this topic, and its relevance when considering strategies to increase donor organ utilisation, the role of MP of the liver as a tool to recondition steatotic livers is discussed. A special focus is given to the possibility of using NMP as a platform for the delivery of drugs that target intracellular lipid metabolism. The pharmacodynamics of the drugs are reviewed, and the benefits of this approach to human donor livers are discussed.

**Chapter 6:** This chapter reports an *in vitro* study that tests the cytotoxicity of a defatting combination of drugs to primary human cells of the liver. After thoughtful examination of the pharmacodynamics of a combination of defatting drugs presented in Chapter 5, primary human cells of the liver are isolated and exposed to these defatting drugs. This preliminary study indicates that the agents are not toxic to primary human hepatocytes, human intrahepatic endothelial cells and cholangiocytes in culture. In addition, steatosis is induced in primary human hepatocytes by supplementation of the perfusate with a combination of saturated and unsaturated free fatty acids. Thereafter, the effectiveness of the drugs in enhancing the

metabolism of intracellular lipid stores, and thus promoting steatosis reversal, is assessed in the fat-laden hepatocytes.

**Chapter 7:** Following investigation of alternatives of pharmacological interventions to rescue steatotic donor organs in Chapter 5; and testing *in vitro* the toxicity of the drugs to primary human cells of the liver in Chapter 6; Chapter 7 describes the results of the delivery of defatting drugs to whole human livers discarded for transplantation undergoing NMP. This first proof of concept study reveals that *ex situ* manipulation of the hepatic lipid metabolism during NMP is feasible. It not only decreases the intracellular lipid content of the cells, it also enhances metabolic support to other intracellular processes. An outstanding impact of this therapeutic can be seen in the biliary system: treated livers not only produced a higher volume of bile, but the quality of the bile also significantly improved (determined based on bile pH). Finally, all of the treated livers reached the Birmingham viability criteria, and therefore would be considered transplantable.

**Chapter 8:** This chapter reviews the current clinical evidence of the effect of MP of the liver on post-transplant biliary complications. Ischaemic-type biliary lesions following LT increases postoperative patient morbidity, and it is frequently associated with graft loss and re-transplantation. The mechanisms underlying the aetiology of this disease are investigated, and the potential protective role of MP is discussed. Subsequently, the studies performed during the development of this thesis are placed within the relevant context and their potential positive effect proposed.



**Chapter 9:** This final chapter comprises a general discussion and conclusion. The contributions of this thesis to the existing scientific knowledge are comprehensively interpreted, and the limitations of the present work are discussed. Finally, the implications of this thesis for future studies are proposed.

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## Chapter 2

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# **PUSHING THE LIMITS: MACHINE PERFUSION OF THE LIVER AS A TOOL TO RECONDITION HIGH-RISK GRAFTS**

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### **Published article**

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Y.L.B. designed the study, performed the literature review, interpreted the data and wrote the manuscript. S.C.A. reviewed critically the manuscript and assisted in editing it. H.M. designed the study, reviewed critically the manuscript, assisted in the interpretation of the data and editing the manuscript.

## **2.1 Abstract**

### **Purpose of the Review:**

Machine perfusion is a novel technology recently introduced in liver transplantation, redefining the current practice of organ preservation and pushing the limits of high-risk liver utilisation. This review highlights the key benefits of machine perfusion over conventional static cold storage, demonstrated in human liver research and clinical transplants.

### **Recent Findings:**

The first clinical trials have demonstrated both safety and feasibility of machine perfusion. The most recent transplant series and results from a randomised trial suggest the technology is superior to static cold storage. The key benefits include extended period of organ preservation, decreased incidence of early allograft dysfunction and reduction of biliary complications. Normothermic liver perfusion allows viability testing to guide transplantability of the highest-risk organs. This technology also provides opportunities for therapeutic interventions to improve liver function and quality in organs that are currently declined for clinical use.

### **Summary:**

Machine perfusion is likely to transform the liver preservation pathway and to improve utilisation of high-risk grafts.



## 2.2 Introduction

A recent report from the British National Health Service identified that 13% of the 1769 patients registered for liver transplantation in the United Kingdom (UK) between 2016 and 2017 died or were removed from the waiting list after becoming too ill for transplantation (1). Similar data are reported from many other countries reflecting that organ shortage in the context of rising incidence of liver disease is a worldwide phenomenon (2). To address shortfalls in supply of standard criteria donors, transplant teams have progressively extended the criteria to accept marginal, high-risk organs (3). Such extended criteria donors (ECD) include donors after circulatory death (DCD), fatty livers, and organs from donors with higher risk behaviour or advanced age with multiple comorbidities (3). ECD grafts are more susceptible to ischaemic injury and detrimental effects caused by static cold storage (SCS) and are associated with an increased rate of early allograft dysfunction, post-transplant biliary complications and recipient mortality (4–6). Consequently, an increasing number of procured livers are being declined for transplantation (7). Steatosis is the leading cause worldwide of livers being discarded (40%), followed by prolonged donor warm ischaemia, poor organ flushing and logistical reasons (8, 9). Confronting the ongoing decline of standard criteria donors, the increased need for transplantation can be achieved only by more successful transplants from marginal grafts and increased utilisation of high-risk livers (10).

The inferior outcomes of ECD grafts have exposed shortcomings of SCS and directed research towards finding superior means of liver preservation. Following encouraging data from pre-clinical experiments and breakthroughs achieved in heart, lungs and kidney preservation, several teams around the world have reported successful transplantation of machine-perfused livers, and this promising technology has attracted the attention of the transplant community worldwide (3, 11–14).

Machine perfusion of the liver (MP) has become a rapidly progressing field. Whilst the initial case series demonstrated the technology is safe and feasible in standard criteria livers, subsequently conducted randomised trials have evaluated its efficacy on a whole spectrum of currently utilised organs (11, 15–19) Table 2.1. In keeping with promising experimental data, the current evidence suggests that MP will be most beneficial when applied to ECD livers.

**Table 2.1: Evidence and benefits of machine perfusion over the static cold storage**

Study endpoint*	Machine perfusion technique				
	Hypothermic non-oxygenated perfusion	Hypothermic oxygenated perfusion <sup>@</sup>	Normothermic preservation	Normothermic reconditioning	Sub-normothermic reconditioning
Safety and feasibility: standard criteria donors	PCS, $n^{\S} = 20$ [ref: <a href="#">33</a> ]	PCS, $n = 8$ [ref: <a href="#">34</a> •]	RCT <sup>&amp;</sup> , $n = 220$ [Nasralla <i>et al.</i> , data submitted; ref: <a href="#">11</a> •, <a href="#">14</a> ]	PCS, $n = 12$ [ref: <a href="#">15</a> •, <a href="#">35</a> ]	PCS, $n = 6$ [ref: <a href="#">42</a> ]
Safety and feasibility: extended criteria donors	PCS, $n = 31$ [ref: <a href="#">19</a> •]	PCS, $n = 25$ [ref: <a href="#">17</a> •, <a href="#">34</a> •, <a href="#">38</a> ]	RCT <sup>&amp;</sup> , $n = 220$ [Nasralla <i>et al.</i> , data submitted; ref: <a href="#">11</a> •, <a href="#">14</a> ]	PCS, $n = 12$ [ref: <a href="#">15</a> •, <a href="#">35</a> ]	PCS, $n = 6$ [ref: <a href="#">42</a> ]
Efficacy: early graft dysfunction	PCS, $n = 31$ [ref: <a href="#">19</a> •, <a href="#">33</a> ]	PCS, $n = 25$ [ref: <a href="#">17</a> •, <a href="#">34</a> •, <a href="#">38</a> ]	RCT <sup>&amp;</sup> , $n = 220$ (Nasralla <i>et al.</i> , data submitted)	PCS, $n = 12$ [ref: <a href="#">15</a> •]	PCS, $n = 6$ [ref: <a href="#">42</a> ]
Efficacy: non-anastomotic biliary strictures	No data available yet	PCS, $n = 25$ [ref: <a href="#">17</a> •, <a href="#">34</a> •]	No data available yet	PCS, $n = 12$ [ref: <a href="#">35</a> ]	No data available yet
Extending the limits: preservation time	Not applicable	No data available yet	PCS, $n = 20$ [ref: <a href="#">11</a> •]	PCS, $n = 12$ [ref: <a href="#">15</a> •]	No data available yet
Graft functional assessment	Not possible	Not possible (no real-time assessment available)	RCT <sup>&amp;</sup> , $n = 220$ (Nasralla <i>et al.</i> , data submitted)	PCS, $n = 12$ [ref: <a href="#">15</a> •, <a href="#">35</a> ]	No data available yet
High-risk liver utilisation	PCS, $n = 31$ [ref: <a href="#">19</a> •, <a href="#">33</a> ]	PCS, $n = 25$ [ref: <a href="#">17</a> •, <a href="#">34</a> •, <a href="#">38</a> ]	No data available yet	PCS, $n = 12$ [ref: <a href="#">15</a> •, <a href="#">35</a> ]	PCS, $n = 6$ [ref: <a href="#">42</a> ]
Therapeutic interventions	No data available yet	No data available yet	No data available yet	No data available yet	No data available yet

PCS, prospective cohort study; RCT, randomised controlled trial; DBD, donation after brain death; DCD, donation after circulatory death. \*The table includes the key evidence published to date in each particular perfusion technique and the static cold storage used as a reference for comparison. <sup>§</sup> $n$  number relates to the largest published series. <sup>&</sup>COPE WP2 trial (ISRCTN 39731134). <sup>@</sup>Multi-centres European randomised controlled trials in progress (HOPE, [NCT01317342](#) and D-HOPE, [NCT02584283](#))

Recently several teams have started programmes to recondition marginal grafts and to explore frontiers of high-risk organ utilisation. The predominantly used perfusion temperatures are hypothermic (8–12 °C) and normothermic (37 °C), although sub-normothermic and rewarming alternatives have been reported (20–23).

The hypothermic perfusate is similar to conventional cold storage preservation fluid, and the perfusion technique simpler and cheaper than normothermic machine perfusion (NMP). It does not pose additional risks of graft loss compared to SCS and can reveal pre-existing organ damage; however, in a clinical setting, it does not generate the data necessary to assess liver function and transplantability (24, 25).

Sub-normothermic perfusion encompasses the temperature range from 12 °C to 35 °C, although the majority of experiments were performed at temperatures close to 21 °C (21, 26, 27). Whilst this perfusion modality delivered promising data in pre-clinical testing, to date, there have not been published data from clinical use. Several teams have investigated graft rewarming, gradually increasing the perfusion temperature from 4 °C to 21 °C with promising results (22, 28, 29). Both these perfusion approaches achieve a partial recovery of liver metabolic function with oxygen requirements that can be met without a dedicated oxygen carrier in the perfusion fluid.

NMP requires a blood-based perfusate and preserves the liver in near-physiological, fully functioning condition, generating real-time data allowing assessment of its viability (15, 30, 31). It can be used as an alternative to SCS and such application has been currently tested in prospective clinical trials across Europe and North America. NMP has arguably the most potential to minimise any deleterious effects of the cold ischaemia, but is less flexible, costlier and logistically more demanding (32). Liver reconditioning provides a more flexible alternative, commencing the perfusion on its arrival at the transplant centre. Several groups

have used this technique to resuscitate graft function prior to transplantation, including our team's work on viability assessment of declined livers (15, 16). It can be applied selectively to grafts with suboptimal macroscopic appearance or livers deemed too high-risk for any other reason.

### **2.3 Extending the Liver Preservation Times**

The progressive detrimental effect of cold ischaemia on graft quality is the fundamental limitation of SCS. Shortening cold ischaemia times (CIT) to the bare minimum has become a key aspect of successful utilisation of ECD organs and many more livers could have been used had the organ arrived at the transplanting centre earlier (33). The benefit of MP on removing the constraints due to CIT has previously been shown in the first-in-man NMP series where the longest preservation time was close to 19 hours (h) (11).

Subsequently, the first randomised trial comparing NMP with SCS performed by the Consortium for Organ Preservation in Europe (COPE WP2 trial, ISRCTN 39731134) identified that clinicians started to exploit this logistic advantage, and livers in the NMP group were preserved for significantly longer compared to SCS (11 h 39 minutes [min] vs. 7 h 21 min,  $p < 0.01$ ). Despite this, the organs suffered from less early allograft dysfunction (12.6% vs. 29.9%;  $p = 0.002$ ; Nasralla *et al.*, data presented at the British Transplant Society Congress in 2017). Recently, Watson *et al.* reported successful implantation of an ECD liver 26 h after procurement; in this example, NMP was used to assess the organ and the transplant was deferred until extra-hepatic malignancy in the recipient was excluded (34). This case report highlights the logistical advantages of NMP and the differences in specification of available devices in terms of the maximum liver preservation times (11, 15, 30, 35). Liberation from the CIT constraints might redefine multiple aspects of current liver transplant practice in the future, including new opportunities for supra-regional graft sharing and super-urgent allocation,

transformation of donor—recipient matching or streamlining operating theatre logistics and transplant teams' workforce management.

The limits of extending the NMP preservation are yet to be defined, though experimental work has demonstrated successful perfusions beyond 24 h (34–37). Whilst 24 h preservation would be sufficient to achieve significant improvements in organ sharing and transplant logistics, MP may preserve livers for significantly longer. Several research groups have reported experiments with 72-h canine and porcine liver perfusions (36, 38). The prolonged perfusion will undoubtedly impose new challenges to overcome. For example, red blood cells (RBCs) have clear biophysical limitations when exposed to sheer stress from the circuit tubing and abrasive mechanical insult from centrifugal or roller pumps, leading to an unavoidable degree of haemolysis (39). To address the problem, our team investigated the feasibility of replacing RBCs with an acellular haemoglobin-based oxygen carrier (HBOC), Hemopure®, in a human model of NMP. Our results demonstrated similar metabolic and flow parameters, whilst the HBOC-perfused livers extracted more oxygen than those perfused with RBCs ( $O_{2ER}$  13.75% vs.  $9.43\% \times 10^5$  per gram of tissue,  $p = 0.001$ ) without increased apoptosis or necrosis, tested in vitro hepatic cell lines (32). Exploring strategies to maintain prolonged perfusion will clearly require intensive research to optimise the perfusate composition and device design, but achieving this goal will open new possibilities for using MP as a tool for therapeutic intervention and regeneration of the highest-risk liver grafts.

## **2.4 Pushing the Limits of High-Risk Organ Utilisation**

While the initial series of machine perfused transplants included essentially standard criteria organs, the technology's greatest benefit is preservation of marginal livers (40). Guarrera *et al.* applied hypothermic perfusion to 31 ECD livers donated after brainstem death (DBD) and, despite their model not providing the organs with oxygen, the authors observed a decreased

rate of biliary complications and hospital stay compared to matched SCS controls (19). Compelling data were reported by the Zurich group using hypothermic oxygenated perfusions (HOPE) of DBD livers (41). Consequently, Dutkowski and colleagues achieved nation-wide adoption of HOPE by the Swiss healthcare for all DCD livers, utilising these marginal organs with superior outcomes. Comparison of their results with matched controls from the Netherlands and UK demonstrated the incidence of non-anastomotic biliary strictures in 25 HOPE-perfused livers compared to 50 SCS matched DCD transplants was significantly lower (0% vs. 22%,  $p = 0.015$ ), together with superior 1-year graft survival (90% vs. 69%,  $p = 0.035$ ) (17). Most recently, the data from the COPE WP2 multi-centre European study enrolling 272 donors (consisting of 194 DBD and 78 DCD organs) showed significantly lower organ discard rate in the normothermic arm (NMP 16 vs. SCS 32 livers;  $p = 0.01$ ; Nasralla *et al.*, data presented at the British Transplant Society Congress in 2017). The data regarding biliary complications and survival at 6-months from this trial are not yet available (Table 2.1).

The development of normothermic perfusion enabled objective assessment of the liver function, advancing the graft selection process, and several teams researched the development of criteria for viability assessment including perfusate pH, bile production, transaminase levels or lactate clearance.

Through pre-clinical experiments, our team observed a close relationship between liver function and perfusate lactate metabolism. The organs able to achieve lactate clearance below 2.5 mmol/L within 120 min of commencing perfusion retained physiological perfusate acid base milieu and did not require any interventions to maintain extended perfusion. Transforming the observations into clinical practice, we proposed composite criteria, based on the lactate clearance and bile production in combination with vascular flows and macroscopic appearance (16). These were applied on a pilot series of six livers declined for transplantation by all the UK transplant centres and subjected to normothermic perfusion. The study enrolled four DCD

and two DBD grafts, commencing perfusion after a period of CIT ranging from 387 to 474 min (15). The liver viability was assessed within a 2-h window, and five of the organs met the criteria and were successfully transplanted. In all recipients, we observed immediate graft function recovery with a short hospital stay, and normalised liver function tests within the first post-transplant month (15). We have consequently conducted the VITTAL clinical trial (Viability Testing and TrAnsplantation of discarded donor Livers; NCT02740608), further testing the boundaries of the highest-risk organ utilisation by criteria principally based on lactate metabolism, extending the assessment period up to 4 h.

NMP provides the opportunity to explore multiple parameters relevant to liver function, and it is still to be determined which can best predict post-transplant outcomes. The Cambridge group advocated graft assessment based on the perfusate transaminases and bile pH (42). The authors observed a significant correlation between the alanine transaminase (ALT) in the perfusate measured after 2 h perfusion and the peak ALT post-transplant levels within the first week (42). In this report, Watson *et al.* also hypothesise that the liver capacity to produce an alkaline bile ( $\text{pH} > 7.4$ ) might be a marker of good cholangiocyte function, possibly allowing selection of organs with a low risk of developing intrahepatic cholangiopathy. If validated, this observation might revolutionise DCD liver utilisation, preventing futile transplantation of grafts with a limited life span. We expect that future organ functional assessment will include more sophisticated methods and markers based on perfusate omics or microRNA analyses (43, 44).

Although the viability testing data have already shown benefits of NMP in high-risk graft utilisation and reduced risk of early graft failure, the evidence that MP improves long-term transplant outcomes is still elusive. A pertinent cohort in which to study this subject are DCD livers. To date, the only clinical evidence comes from retrospective observations published by the Zurich and Groningen groups (17, 41, 45).



The experimental evidence from normothermic perfusion suggests a protective effect of peribiliary glands (46–48). The evaluation of the biliary complication data from the randomised COPE trials is eagerly awaited.

## **2.5 New Frontiers and Therapeutic Interventions during Machine Perfusion**

Designing a MP clinical trial powered to demonstrate differences in post-transplant graft or patient survival is very challenging and researchers often use a validated surrogate endpoint as a substitute. A difference in the post-transplant transaminase levels, incidence of delayed graft function, and markers proving less ischaemia-reperfusion injury have been a frequent reported endpoint with many MP series (11, 15, 17, 28, 40, 49). The circulating perfusate in the liver itself prevents accumulation of succinate and other metabolic products and removes the debris and necrotic or apoptotic cells, likely decreasing the post-reperfusion transaminases and having a beneficial effect on the graft (19, 50). The major benefit is, however, the oxygenation preventing the damage caused by ischaemia and anaerobic metabolism (50). The pre-clinical research gathered mechanistic evidence of multiple aspects of cellular metabolism pathways influenced by MP with several teams focused in particular on energy metabolism and mitochondrial function (51). The HOPE perfusion was shown to induce mitochondrial function with down-regulation of the respiratory rate, associated with adenosine tri-phosphate synthesis. Such 60 to 120-min duration perfusion achieves recovery of the liver energy resources together with prevention of mitochondrial reversal flow of electrons during the organ re-warming, decreasing production of reactive oxygen species mitigating activation of the inflammatory processes involved in the reperfusion injury (52, 53).

Despite an extensive knowledge on a cellular level of the mechanisms of hepatic function protection during HOPE, NMP has the advantage of allowing the exploration of targeted therapeutic interventions analogous to conventional medical approaches. For example, to

combat the anticipated risks of bacterial contamination and overgrowth, antibiotics are universally added to the normothermic perfusate fluid composition (30, 32). Targeted antibiotics added to the perfusate for livers from positive culture donors might be an easy intervention to improve transplantability of organs from donors with infections. A proof of concept with NMP antiviral pre-treatment during MP in a porcine model was recently reported by the Toronto group (54). The same group also explored strategies to further enhance the protective MP mechanisms in a sub-normothermic porcine DCD liver model, enriching the perfusate with anti-inflammatory drugs (alprostadil, n-acetylcysteine, carbon monoxide and sevoflurane). This intervention significantly lowered the perfusate levels of aspartate aminotransferase, interleukin 6, tumour necrosis factor alpha and galactosidase, and increased interleukin 10 levels compared to the untreated controls. MP itself reduces activation of the post-reperfusion inflammation cascade, and despite the improvements in reperfusion injury markers not achieving statistical significance, this concept to enhance the protective mechanisms merits further research (52, 53).

Another frequent reason for discarding donor livers is a suboptimal flushing. Addition of a thrombolytic agent together with the standardly included heparin might improve graft circulation without increasing the bleeding risks for the organ recipient (31, 55).

Steatotic livers are the largest group of poorly utilised organs, and resuscitation of fatty livers is an important goal whilst combating the growing obesity epidemic (56). The concept of pharmacological intervention to reverse steatosis during NMP has been explored by several teams including our own. We performed NMP on severely steatotic donor human livers, exposing the organs to a combination of defatting drugs. We observed solubilisation of the liver fat, commencing within 3 h, and continuing until the end of 24-h perfusions. Although it is unclear whether removal of fat from a viable liver during NMP would be relevant in improving its post-transplant function and outcome, the treated organs showed significantly

better metabolic parameters compared to matched controls, and the histological improvement became apparent after only 6 h. A potential shortfall of MP with metabolically active livers might be re-circulation of harmful or toxic metabolites (57). In the described experiment the mitochondria fatty acids  $\beta$ -oxidation increased ketone production and continuously increased the perfusate apolipoprotein and cholesterol levels. Removal of the metabolism by-products from the device circuit is an interesting issue requiring more research.

The perfusion model of steatotic livers also demonstrates that different graft categories may benefit from different MP modalities. Cooling the hepatic fat leads to changed lipid consistency, increasing its droplet volume which ultimately compromises the liver microcirculation. The optimal strategy to minimise any post-procurement damage might be minimising exposure to the cold by normothermic preservation. Following a period of SCS, however, the disturbed hepatic microcirculation makes subsequent machine resuscitation and perfusion processes challenging and the optimal temperature and combination of different approaches is yet to be determined.

The feasibility of the therapeutic interventions discussed has been demonstrated by animal or proof of concept human research. In the near future, MP will be studied as a method to deliver cell-based and small molecule therapies, aimed at improving the condition of high-risk liver grafts, following the emerging evidence showing efficacy of these novel concepts in promoting organ regeneration (58–61).

## **2.6 Conclusion**

MP is a rapidly progressing field which is likely to change multiple aspects of liver preservation and transplantation practice in the future. This superior organ preservation mode has already shown benefits by enabling functional liver assessment with normothermic perfusion or reduction of non-anastomotic biliary strictures by hypothermic perfusion. MP looks likely to

set new limits for organ preservation times, increase utilisation of the highest-risk DBD grafts and improve long-terms outcomes in DCD livers. The perfusion procedure will provide an opportunity for liver regeneration and therapeutic intervention. Different type of livers may benefit from different perfusion strategies or their combination.

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## Chapter 3

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# COMBINED HYPOTHERMIC AND NORMOTHERMIC MACHINE PERFUSION IMPROVES FUNCTIONAL RECOVERY OF EXTENDED CRITERIA DONOR LIVERS

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### Published article

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Y.L.B. designed the experiment, performed the experiment, interpreted the data and wrote the manuscript. R.W.L. assisted in some of the experiments. A.Sc. assisted in some of the experiments and in the analysis of the data. L.W. assisted with the laboratory tests. A.Sm. provided the pharmacological support for the experiments. J.A. assisted in some of the experiments. R.H.B. assisted in editing the manuscript. D.A.H.N. and S.H. performed the histopathological assessment of the samples. M.T.PR.P., D.F.M. offered surgical support for the experiments. S.C.A. designed the experiments, assisted in the interpretation of the data and editing the manuscript. H.M. designed the experiments, assisted in the interpretation of the data and editing the manuscript.

### 3.1 Abstract

**Background:** Hypothermic oxygenated perfusion and normothermic perfusion are seen as distinct techniques of *ex situ* machine perfusion of the liver. We aimed to demonstrate the feasibility of combining both techniques and whether it would improve functional parameters of donor livers into transplant standards.

**Methods:** Ten discarded human donor livers had either 6 hours of normothermic perfusion ( $n = 5$ ) or 2 hours of hypothermic oxygenated perfusion followed by 4 hours of normothermic perfusion ( $n = 5$ ). Liver function was assessed according to our viability criteria; markers of tissue injury and hepatic metabolic activity were compared between groups.

**Results:** Donor characteristics were comparable. During the hypothermic perfusion phase, livers down-regulated mitochondrial respiration (oxygen uptake,  $p = 0.04$ ;  $p\text{CO}_2$  perfusate,  $p = 0.04$ ) and increased ATP levels 1.77-fold. Following normothermic perfusion those organs achieved lower tissue expression of markers of oxidative injury (4-hydroxynonenal,  $p = 0.008$ ; CD14 expression,  $p = 0.008$ ) and inflammation (CD11b,  $p = 0.02$ ; vascular cell adhesion molecule 1,  $p = 0.05$ ) compared with livers that had normothermic perfusion alone. All livers in the combined group achieved viability criteria, whereas 40% (2/5) in the normothermic group failed ( $p = 0.22$ ).

**Conclusion:** This study suggests that a combined protocol of hypothermic oxygenated and normothermic perfusions might attenuate oxidative stress, tissue inflammation and improve metabolic recovery of the highest-risk donor livers compared to normothermic perfusion alone.

### 3.2 Introduction

The rising incidence of liver disease, in combination with changes in organ donor demographics, has increased reliance on extended criteria donor (ECD) livers for transplantation globally (1, 2). Although these organs have inferior outcomes compared to standard criteria livers, their use is deemed necessary to control the waiting list mortality (3–5). Current utilisation of ECD livers remains relatively low mainly when risk factors concur. For example, in the United Kingdom 17% of the procured livers are currently not transplanted (6). *Ex situ* machine perfusion is a novel preservation method developed to protect organs from the detrimental effects of ischaemia during static cold storage (SCS). Potential beneficial protective mechanisms of machine perfusion have been demonstrated for both hypothermic and normothermic perfusion techniques during pre-clinical experiments and pilot clinical studies (7–9).

Hypothermic oxygenated perfusion (HOPE) may permit mitochondrial functional recovery, increasing cellular adenosine triphosphate (ATP) levels, and mitigate the injury to the tissue that occurs during rewarming (9). Whilst there is a mounting evidence of the protective effect of HOPE on the liver graft quality, evidence regarding its value in viability testing still under investigation. Hoyer et al. reported a correlation between perfusate transaminases content and its post-transplant levels suggesting that hepatocellular injury could potentially be assessed also outside of normothermia (10, 11). Normothermic machine perfusion (NMP) of the liver enables metabolism at physiological temperature and therefore facilitates functional assessment (12). Several groups including our own have demonstrated that viability assessment by NMP can be used to select transplantable livers from the pool of currently discarded organs (12, 13). There is no convincing data, however, to show that NMP improves the quality of ECD organs injured by cold ischaemia storage. The key benefit of NMP might be in preventing any deterioration of

liver quality from the time of commencing the perfusion and by providing a snapshot of the organ injury occurred, with the opportunity to assess multiple functional parameters.

Data from our research NMP perfusions showed that a proportion of poor-quality livers exposed to prolonged cold storage do not recover their function and fail our viability criteria. Whilst hypothermic and normothermic perfusion were developed as distinct strategies, we hypothesised that HOPE might be seen as a beneficial therapeutic intervention by restoring liver metabolism prior to a period of normothermic perfusion which permits liver viability testing. The present study aimed to assess the feasibility of a protocol combining HOPE with NMP and to investigate its potential benefits over NMP alone.

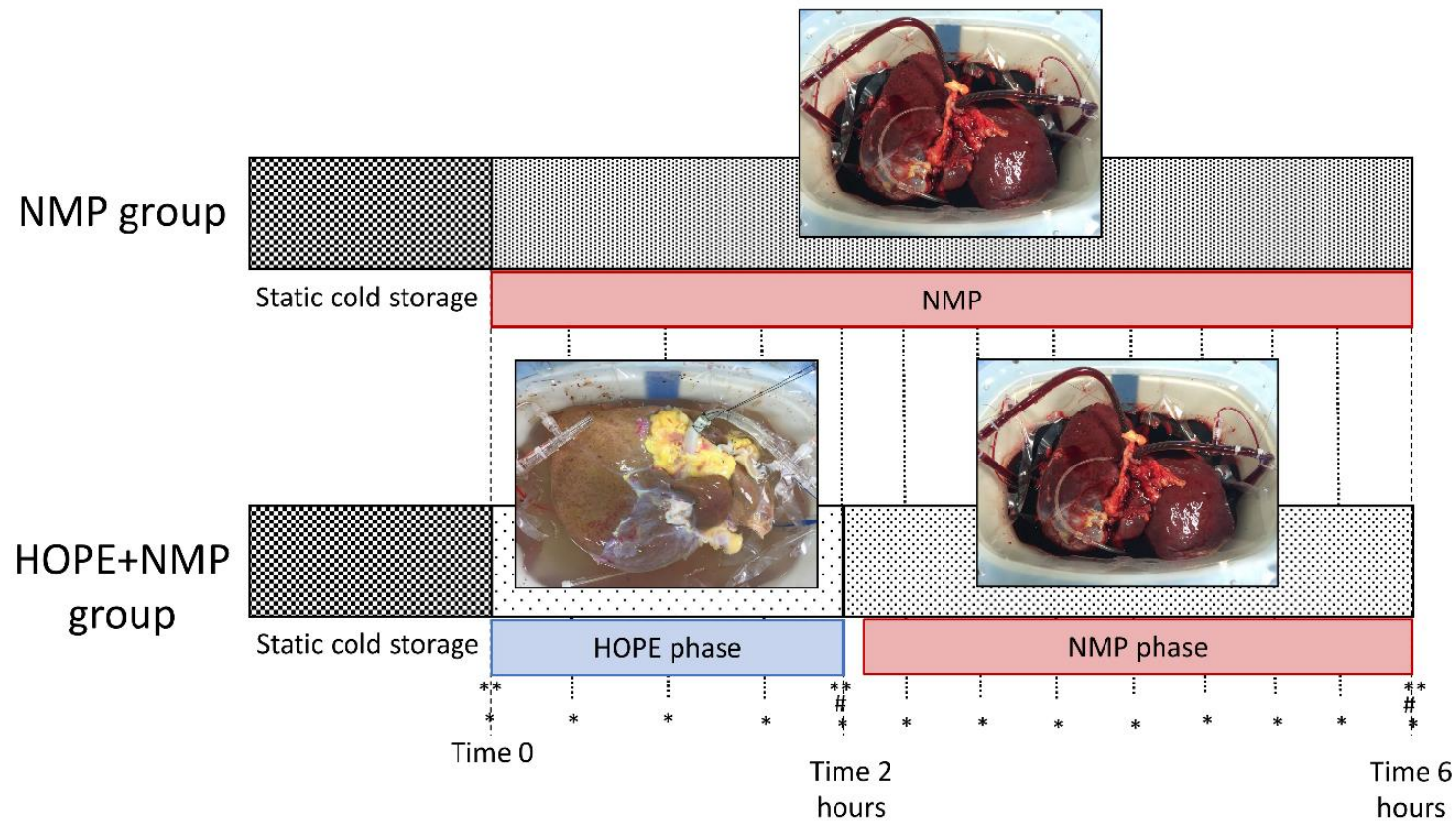
### **3.3 Materials and methods**

#### **3.3.1 Study design**

The study was designed to compare two perfusion strategies to restore function of high-risk ECD livers, following a period of SCS, within the certified 6-hours timeframe allowed by the perfusion device used.

The study endpoints were evaluation of hepatocellular injury and liver function assessment. Ten discarded human donor livers were consecutively assigned to two study groups, each consisting of 5 organs and all perfused for 6 hours. Livers in one group were exposed to normothermic perfusion alone (NMP group, performed first), whilst the other group underwent 2 hours of hypothermic oxygenated perfusion, followed by 4 hours of normothermic perfusion (HOPE+NMP group). The study design and sampling protocol is shown in Figure 3.1.

**Figure 3.1: Study design.**



Discarded human livers were subjected to our routine organ procurement procedure and then cold flushed and cold stored. The organs were allocated randomly in two experimental groups of end-ischaemic machine perfusion. The NMP group was subjected to 6 hours NMP at 37 °C; and, the HOPE+NMP group, was subjected to 2 hours of hypothermic oxygenated perfusion (HOPE) followed by 4 hours of NMP. Menghini and wedge biopsies were collected at time 0 and 6-hours (\*\*) and immediately fixed in formalin or snap-frozen in liquid nitrogen. The HOPE+NMP group had an extra liver biopsy taken at 2 hours. Blood gas analysis was carried out and perfusate was sampled at 30 min time intervals throughout (\*). In addition, bile was collected and weighted at time 4 and 6 hours (#).

### **3.3.2 Source of discarded human livers**

All included livers were procured with the intention of transplantation according to the National Organ Retrieval Service standards. The organs were declined for clinical use by all the UK liver transplant centres and subsequently offered for research purposes. The livers were preserved in University of Wisconsin fluid under standard clinical practice of SCS prior to commencing perfusion. The study ethical approval was obtained by the London-Surrey Borders National Research Ethics Service and the National Health Service Blood and Transplant Ethics Committee (references 13/LO/1928 and 06/Q702/61, respectively).

### **3.3.3 Liver perfusion procedure**

The liver preparation for the perfusion was carried out using a standard clinical back-table procedure as described elsewhere (14). The cystic duct was ligated, and coeliac trunk, portal vein and bile duct cannulated with a 12 French biliary drain. Before commencing machine perfusion, the liver was flushed with two litres of 5% Glucose solution, placed into the device reservoir and the cannulas connected to the perfusion circuits. The Liver Assist device (Organ Assist, Groningen, The Netherlands) provides dual perfusion of the hepatic artery and portal vein, in a semi-closed circuit, by two rotatory pumps to produce pulsatile and non-pulsatile flows respectively (15). The perfusate temperature and perfusion pressures were set by the operator, and the measured flow rates and calculated resistances, shown on the device's display in real-time, recorded every 30 minutes. Oxygen was supplied via a Sechrist air/oxygen blender (S3500CP-G, Inspiration Healthcare Ltd., Leicester, UK) with the fraction of inspired oxygen ( $\text{FiO}_2$ ) and air flow adjusted as specified below.

### **3.3.4 Hypothermic oxygenated perfusion**

The HOPE perfusion was performed via portal vein only, using three litres of Belzer MPS®

University of Wisconsin Machine Perfusion Solution (Bridge to Life Ltd, EU), with the temperature set at 10 °C. The target flow was 0.1 mL/min/g of liver with a maximum pressure of 3 mmHg. The target oxygen perfusate pressure was 80–100 kPa. After 2 hours of HOPE, the perfusion was stopped, and the liver temporarily placed on ice. The system was then drained and subsequently refilled with the NMP perfusion solution. The hepatic artery and common bile duct were cannulated and the NMP perfusion commenced. The perfusion fluid exchange until the start of NMP took on average 20 minutes.

### **3.3.5 Normothermic machine perfusion**

The perfusion fluid for the NMP consisted of 1000 mL (4 units) of an acellular, polymerised bovine haemoglobin-based oxygen carrier Hemopure<sup>®</sup> (Hemoglobin<sup>®</sup> Oxygen Therapeutics LLC, Cambridge, USA) complemented with human albumin solution and additional supplements as described in Supplementary Table S3.1. For superior oxygen delivery, rheological characteristics and research logistic, this solution became our preferred perfusate (16).

The target of flow was 0.25 mL/min/g liver tissue on the arterial side and 0.75 mL/min/g liver tissue in the venous circuit. To achieve these flows, perfusion pressures on the device were adjusted between 30–50 mmHg (mean pressure) on the arterial side and 8–10 mmHg on the portal vein. The temperature was initially set at 20 °C and increased incrementally to 37 °C within 30 minutes of starting NMP. The target perfusate oxygen pressure was 40 kPa.

### **3.3.6 Samples and data collection protocol**

Liver biopsies were taken before commencing the perfusion ( $t = 0$ ), after finishing the HOPE perfusion ( $t = 2$  hours), and on completion of NMP ( $t = 6$  hours; Figure 3.1). Biopsies were immediately either placed in formalin or snap-frozen in liquid nitrogen for subsequent analysis. The biopsy at the end of the HOPE phase was used for ATP assessment only, and all other

analyses were done on biopsies from the beginning and end of the perfusion. The perfusate was sampled every 30 minutes throughout the perfusion in both groups and analysed immediately for blood gases, or snap-frozen and stored for later analyses. Bile produced during the perfusion was collected via a 12 French silicon tube inserted into the common bile duct and weighed at 4 and 6 hours of machine perfusion in both groups. The density of bile was considered 1 g/mL and this was normalised for liver mass.

### **3.3.7 Assessment of liver function**

Arterial and venous perfusates were assessed using a Cobas b 221 (Roche Diagnostics, Indianapolis, USA) point of care system blood gas analyser. Parameters included pO<sub>2</sub> and pCO<sub>2</sub>, pH, base excess, bicarbonate, O<sub>2</sub> saturation, haemoglobin, haematocrit, sodium, potassium, chloride, calcium, glucose and lactate concentrations.

Oxygen uptake was calculated during the HOPE perfusion as the difference between the oxygen inflow minus the outflow in kPa, corrected by liver weight and litres of perfusate. During NMP the oxygen consumption was calculated from the difference between the oxygen content after the oxygenator and the return into the oxygenator in the venous circuit. Oxygen content was calculated as the sum of the free dissolved oxygen fraction to the haemoglobin-bound oxygen fraction (equation in the supplementary methods) as described elsewhere (17).

The viability of the organ was assessed at the end of the perfusion by our unit's clinical criteria, based on perfusate lactate levels falling to concentrations of less than 2.5 mmol/L within 6 hours, in combination with evidence of bile production, stable vascular flows and homogeneous parenchymal perfusion (12).

### **3.3.8 Histopathological assessment of hepatocyte injury**

Menghini needle and wedge biopsies obtained prior to perfusion and at the end were fixed in formalin, processed and embedded in paraffin. Thereafter, 4 µm sections were cut and stained



with haematoxylin and eosin and periodic acid-Schiff (PAS). Haematoxylin and eosin sections were semi-quantitatively graded for ischaemic-type coagulative necrosis, large and small droplet macrovesicular steatosis and pre-existing acute or chronic liver disease. The PAS-stained sections were scored for percentage of hepatocytes depleted of glycogen, and the variation between the beginning and the end of the perfusion compared across groups. Histological assessment was conducted by an experienced liver transplant pathologist without prior knowledge of the designated perfusion category or outcome.

### **3.3.9 Immunohistochemical assessment of oxidative stress and tissue inflammation**

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections to assess surrogate markers of oxidative injury and tissue inflammation.

For oxidative injury we assessed expression of: (1) uncoupling protein 2, a mitochondrial inner membrane protein that uncouples the electron transport chain from oxidative phosphorylation. Elevated uncoupling protein 2 expression is associated with increased reactive oxygen species (ROS) production (17,19); (2) 4-hydroxynonenal (4-HNE) as a marker of cell membrane phospholipid peroxidation (18).

For the assessment of tissue inflammation, the following markers were analysed: (1) cluster of differentiation 14 (CD14), a lipopolysaccharide receptor which is part of the toll-like receptor 4 signalosome. It is essential for activation of the toll-like receptor 4 via the recognition of ligands such as damage-associated molecular patterns known to be upregulated during ischaemia reperfusion injury (18, 19). (2) CD11b is an integrin on the surface of leukocytes; upregulation on its expression indicates activation of the cells by substances including ROS. (3) The vascular cell adhesion molecule 1 (VCAM-1) expression is upregulated on vascular endothelial cells and Kupffer cells when activated by ROS and proinflammatory cytokines during ischaemia reperfusion injury (20). All primary antibodies were detected using specific

ImmPRESS™ Excel Amplified HRP Polymer Staining Kit specific to the respective mouse or rabbit immunoglobulin isotype. A list of primary antibodies and the dilution it was used is provided in the supplementary material.

### **3.3.10 Immunohistochemistry quantitation**

Four pictures of each section excluding the edges were randomly selected for analysis (x400 magnification). For UCP-2, 4-HNE, CD14 and CD11b, a semiquantitative scoring system, the modified immunoreactive score (IRS) (21), was obtained by multiplying the score for intensity (0: no colour reaction; 1: mild reaction; 2: moderate reaction; 3: intense reaction) and distribution (0: no positive cells; 1: < 10% positive cells; 2: 10–50% positive cells; 3: 51–80% positive cells; 4: > 80% positive cells) to obtain a final score between 0 and 12. Change in the overall tissue expression of staining ( $\Delta$ IRS), was determined by subtracting the IRS scores after 6 hours of perfusion from the score prior to perfusion: negative values indicated a decrease and positive an increase in the expression of the staining.

VCAM-1 tissue expression was assessed by image analysis using an established system of colour differentiation (ImageJ, U. S. National Institutes of Health, Bethesda, USA) and the variation in the percentage of the positive area of staining ( $\Delta\%$ VCAM-1) over the time was compared between groups.

### **3.3.11 Assessment of tissue adenosine triphosphate concentration**

Quantification of ATP levels was done by homogenisation of liver tissue. With concentration determined using the ATP Bioluminescent Assay kit (FLAA, Sigma-Aldrich Inc, St Louis, USA). More details are provided in the supplementary material.

### **3.3.12 Statistical analysis**

Continuous variables were expressed as median with interquartile range (IQR) and categorical

variables as absolute number with percentage frequencies. Comparisons between groups were performed using Fisher's exact test for categorical variables, the Mann–Whitney  $U$  test for independent continuous variables and the Wilcoxon signed-rank test for repeated measurements over time on the same sample. The statistical level of significance was set at  $p < 0.05$ . GraphPad Prism (version 6.04 for Windows, GraphPad Software, La Jolla, USA) software was used for all statistical analyses and graph creation.

### **3.4 Results**

#### **3.4.1 Donor demographics and discarded liver characteristics**

Seven livers (70%) were from donors after circulatory death (DCD). The median donor age of the entire cohort was 52 (IQR: 38–54) years and the body mass index 25 (21–31) kg/m<sup>2</sup>. Median cold ischaemia time was 510 minutes (446–682) for DCD, and 491 minutes (454–586) for donors after brainstem death (DBD). The median donor risk index (DRI) was 2.2 (1.9–2.6). There were no significant differences in donor demographics between the groups as shown in Table 3.1 and they had similar median DRI (NMP 2.4 vs. HOPE+NMP 2.5,  $p = 0.78$ ). There was a trend towards NMP livers having shorter SCS preservation times for DCD (449 vs. 682 minutes,  $p = 0.09$ ). The detailed donor and liver data is presented in Tables 3.1 and 3.2.

**Table 3.1: Donor demographics, liver characteristics and machine perfusion parameters.**

Liver number	NMP 1	NMP 2	NMP 3	NMP 4	NMP 5	HOPE+ NMP 1	HOPE+ NMP 2	HOPE+ NMP 3	HOPE+ NMP 4	HOPE+ NMP 5
<b>Donor information</b>										
Age	70	36	50	25	60	54	50	54	38	55
Donor type	DCD	DCD	DBD	DCD	DCD	DCD	DBD	DBD	DCD	DCD
Sex	Male	Male	Male	Male	Male	Male	Female	Female	Male	Female
Height (cm)	165	181	187	175	189	179	158	170	183	160
Bodyweight (kg)	80	70	90	82	75	123	60	87	85	90
Body mass index (kg/m <sup>2</sup> )	29	21	25	26	21	38	24	30	25	35
US - Donor risk index	3.2	2.6	1.6	2.2	2.2	2.5	2.0	1.9	1.8	3.0
UK - donor liver index	4.0	2.1	0.9	2.1	27.4	16.3	6.9	1.2	3.1	4.9
ET - donor risk index	3.3	2.2	1.7	1.9	2.4	2.9	2.2	1.9	2.3	2.8
Peak ALT (IU/L)	168	17	44	476	137	189	14	271	37	741
Days on ventilator	1	5	1	3	8	5	2	2	4	4
Co-morbidities and risk history	HTN	Smoker	Alcohol misuse	Smoker, alcohol misuse	Smoker	Diabetes (type 2) HTN	Smoker, Alcohol misuse	Diabetes (type 1), Smoker	Active Smoker	HTN
Cause of death	ICH	ICH	ICH	Trauma	Trauma	HBD	ICH	ICH	ICH	HBD
<b>Liver characteristics</b>										
Liver weight (g)	2208	2218	2380	1998	1555	2600	1838	2060	1753	1935
Donor WIT (min)	24	20	NA	10	17	31	NA	NA	42	8
CIT (min)	384	453	464	612	446	497	682	491	660	510
Retrieval location	Regional	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal
Reason for clinical rejection	Steatosis	Poor flush	Donor cancer	High ALT, poor flush	Donor cancer	Poor flush	Steatosis	Steatosis	Poor flush	Steatosis, poor flush
Large MaS	60%	0%	0%	0%	0%	10%	0%	15%	3%	30%
<b>Machine perfusion parameters</b>										
Lactate (mmol/L)										
Highest	9.6	20.0	10.3	10.4	9.0	9.1	8.9	10.4	4.6	7.8
Lowest	4.1	8.8	0.3	1.4	0.6	0.6	1.1	1.8	0.2	0.8
At 6-hour	5.3	11.6	0.3	1.6	1.4	0.6	1.1	1.8	0.2	0.8
Total Bile production (g)	0.0	0.0	17.6	26.0	38.0	57.0	0.0	0.0	15.9	24
Mean Arterial flow (mL/min)	535	256	760	529	616	292	299	234	152	398
Mean Portal vein flow (mL/min)	1188	926	1500	1015	1020	1412	1523	1602	1406	1582
Mean liver mass perfusion(mL/g/min)	0.78	0.53	0.95	0.77	1.05	0.66	0.99	0.89	0.89	1.02
Viability criteria	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

**Abbreviations:** NMP: normothermic machine perfusion of the liver; HOPE: hypothermic oxygenated perfusion of the liver; DCD: donation after circulatory death; DBD: donation after brain death; US: United States; UK: United Kingdom; ET: Eurotransplant; ALT: alanine aminotransferase; HTN: hypertension; ICH: intracranial haemorrhage; HBD: hypoxic brain damage; NA: not applicable; WIT: warm ischaemic time; CIT: cold ischaemia time; MaS: macrovesicular steatosis (paraffin sections).

**Note:** Donor warm ischaemic time was defined as the interval between the systolic blood pressure less than 50mmHg or/and arterial oxygen saturation to less than 70% to commencing the aortic cold perfusion in the donor.

**Table 3.2: Donor demographics, liver characteristics and machine perfusion data.**

	NMP ( <i>n</i> = 5)	HOPE+NMP ( <i>n</i> = 5)	<i>p</i> -value
<b>Donor information</b>			
Age, years	55 (43–65)	54 (46–54)	0.84
DCD livers	4 (80%)	3 (60%)	0.50
Sex, male	5 (100%)	2 (40%)	0.17
Height (cm)	184 (173–188)	179 (169–181)	0.19
Bodyweight (kg)	78 (73–85)	90 (87–106)	0.39
Body mass index (kg/m <sup>2</sup> )	23 (21–27)	35 (30–37)	0.08
Donor risk index	2.4 (1.9–2.8)	2.5 (2.2–2.7)	0.78
UK donor liver index	3.1 (1.5–15.7)	4.9 (4.0–10.6)	0.89
ET donor risk index	2.3 (1.9–2.9)	2.8 (2.6–2.8)	0.80
Peak ALT (IU/L)	68 (44–137)	189 (37–271)	0.24
Days on ventilator	3 (1–5)	4 (2–4)	0.89
<b>Liver characteristics</b>			
Liver weight (g)	2208 (1998–2218)	1935 (1838–2060)	0.87
Donor warm ischaemic time (minutes)	20 (16–22)	31 (19–36)	0.42
Cold ischaemia time (minutes), DCD	449 (423–532)	682 (586–708)	0.09
Cold ischaemia time (minutes), DBD	454 (454)	586 (490–682)	0.57
Macrovesicular steatosis (%)	0 (0–30)	10 (1–22)	0.98
<b>Machine perfusion parameters</b>			
Lactate (mmol/L)			
Highest	10.3 (9.6–10.4)	8.9 (7.8–9.1)	0.14
Lowest	1.4 (0.6–4.1)	0.8 (0.6–1.1)	0.22
Last	1.6 (1.4–5.3)	0.8 (0.6–1.1)	0.17
Total Bile production (g)	18 (0–32)	16 (0–40)	0.82

**Abbreviations:** NMP: normothermic machine perfusion of the liver; HOPE: hypothermic oxygenated perfusion of the liver; DCD: donation after circulatory death; UK: United Kingdom; ET: Eurotransplant; ALT: alanine aminotransferase; DBD: donation after brain death.

**Note:** Continuous variables presented as median and interquartile range, dichotomous variables as absolute number and percentage. Donor warm ischaemic time was defined as the interval between the systolic blood pressure less than 50mmHg or/and arterial oxygen saturation to less than 70% to commencing the aortic cold perfusion in the donor.

### 3.4.2 Vascular flows

Portal flow patterns and end-perfusion volumes differed between the groups. During the initial 60 minutes of the NMP group perfusion the livers' portal flow rate increased rapidly and plateaued after 180 minutes. For the HOPE+NMP group steady, low volume flow rates approximating 300 mL/min were achieved during the HOPE phase. The flow rate subsequently increased for the first 2 hours of rewarming during the NMP phase. The median flow rates in the HOPE+NMP group exceeded the NMP group after 210 minutes and continued to increase until the end of the perfusion (1300 vs. 1930 mL/min,  $p = 0.03$ ). In contrast, the arterial flows in the NMP livers increased more rapidly during the rewarming phase and remained higher throughout the perfusion (365 vs. 732 mL/min,  $p = 0.09$ ), while the vascular resistance patterns and levels were similar. The detailed data are presented in Figure 3.2 and Table 3.1.

### 3.4.3 Mitochondrial function and oxygen consumption

During the HOPE phase, livers demonstrated a decrease in the oxygen uptake rate and CO<sub>2</sub> release in the perfusate. The oxygen uptake during the HOPE phase peaked within the initial 30 minutes (0.009 [0.005–0.010] kPa/gram liver/litre perfusate), then declined steadily (0.006 [0.001–0.007] kPa/gram liver/litre perfusate) until the end of the 2-hour perfusion ( $p = 0.04$ ). There was a concomitant decrease in the median partial pressures of carbon dioxide dissolved in the perfusate from time 0 to 2 hours of hypothermic perfusion (1.27 [1.17–1.86] to 0.69 [0.57–0.82],  $p = 0.04$ ). This change in mitochondrial respiration was associated with a median 1.77-fold (range 1.33–3.89) increase in tissue ATP levels.

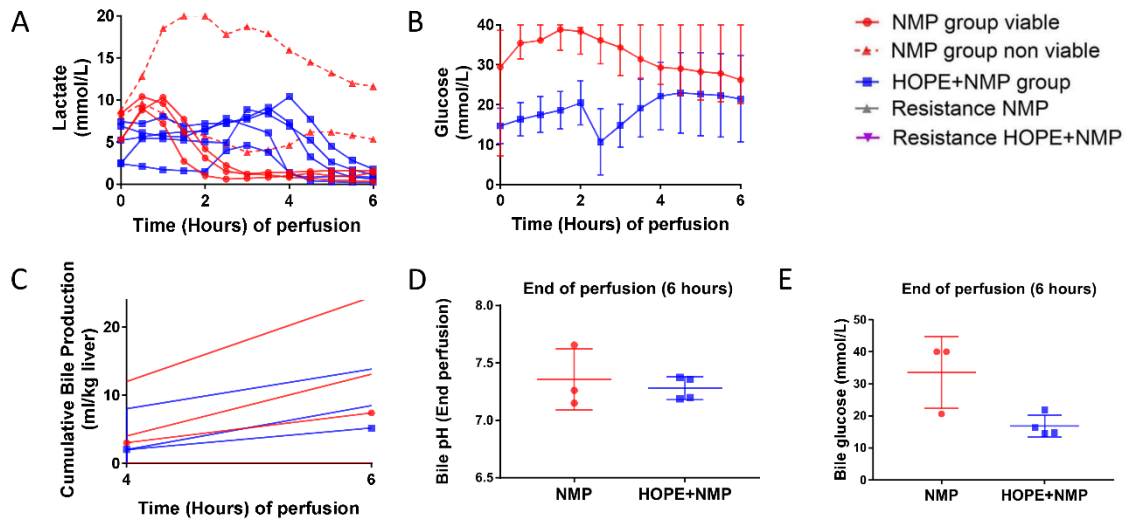
The oxygen consumption in the NMP group increased sharply within the initial 60 minutes with distinct patterns in viable compared to non-viable groups (Figure 3.2). In the HOPE+NMP livers, oxygen consumption increased slower during the normothermic phase and was maximal after 2 hours. At the end of the perfusion, the oxygen consumption in viable NMP livers was

similar to livers that had HOPE ( $p = 0.19$ ).

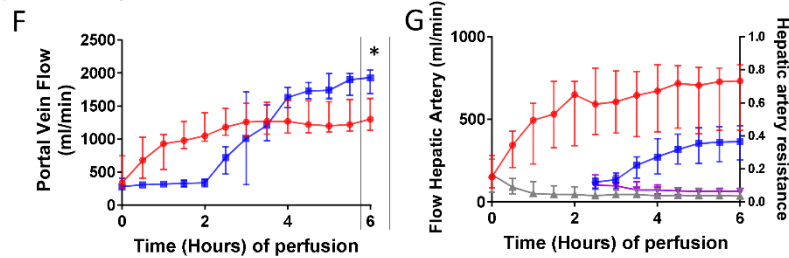
Both groups had similar ATP stores at the end of the 6 hours' perfusion ( $p = 0.31$ ), however there was a significant difference in the incremental rate between viable and non-viable livers in the NMP group (NMP viable 2.53-fold [2.40–10.9]; NMP non-viable 1.10-fold [1.08–1.15]; HOPE+NMP 2.67-fold [2.00–6.65],  $p = 0.05$ ). Data is shown in Figure 3.2.

**Figure 3.2: Perfusion parameters.**

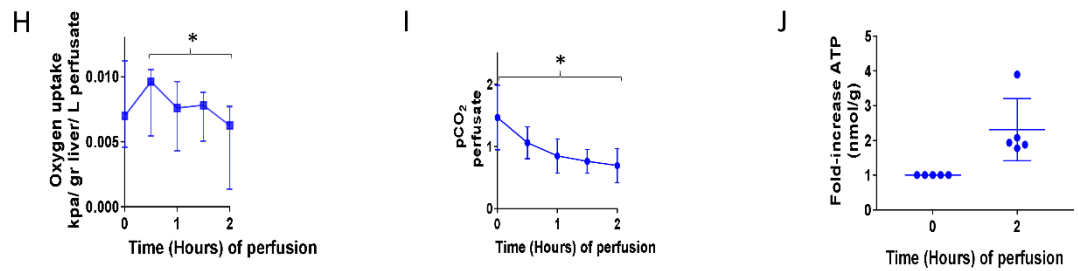
(1) Liver function assessment



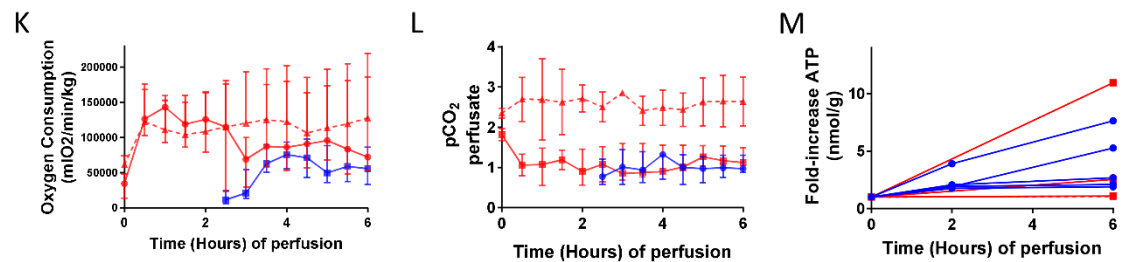
(2) Vascular parameters



(3) HOPE phase and mitochondria function



(4) Energy metabolism along the perfusion





**Figure 3.2: Perfusion parameters.** Section 1: Liver functional assessment- Panel A: Perfusate lactate concentration (mmol/L) - measured throughout the perfusion. Here the NMP group was stratified in the viable livers –continuous red lines- (60%,  $n = 3$ ) or non-viable livers –dotted red lines- (40%,  $n = 2$ ). Blue lines represent the HOPE+NMP group livers. Panel B: Perfusate glucose concentration (mmol/L) - Data presented as median and IQR. Panel C: Cumulative bile production for individual livers after 4 and 6 hours of perfusion corrected for liver weight in kilograms. Panel D: Bile pH at 6 hours of perfusion was comparable between the study groups, as was glucose (Panel E). Section 2: Vascular parameters of the perfusion. Panel F: Portal vein flow rate in mL/min showing that flow increased steadily in the NMP group plateauing after 2 hours. For HOPE+NMP it remained low and stable during the HOPE phase increasing during the rewarming phase and achieving higher rates at the end of the perfusion compared with NMP alone. Panel G: Hepatic artery (HA) flow rate expressed in mL/min plotted on the right Y axis and hepatic artery vascular resistance in mmHg/mL/min/kg liver plotted on the left Y axis. Despite similar resistances at the end of the perfusion, the HA flow reached higher values at the end of the perfusion in the NMP group. Section 3: HOPE phase and mitochondrial function. Panel H: Oxygen uptake during the HOPE phase of the HOPE+NMP group ( $\Delta\text{inflowO}_2\text{-outflowO}_2$ ), expressed as median and IQR. Panel I:  $\text{pCO}_2$  released in the perfusate decreased steadily throughout the hypothermic perfusion phase. Panel J: Tissue ATP levels (nmol/gram), time 2-hours normalised for the concentration in time 0 and expressed as fold increase. Data presented as median and minimum/maximum. Section 4: Energy status during perfusion. Panel K: Oxygen consumption during NMP phase for both groups, NMP stratified according to viability criteria the continuous red line representing viable and the dotted red line representing non-viable respectively. Data presented as median and IQR. Oxygen consumption increased sharply within the initial 60 minutes of NMP whereas the HOPE+NMP livers presented a slower increase in oxygen consumption during the normothermic phase. Panel L:  $\text{pCO}_2$  released in the perfusate during the NMP phase showing that figures were similar between the viable livers of the NMP group and the HOPE+NMP group. Non-viable livers from the NMP group presented with higher perfusate  $\text{pCO}_2$  levels throughout the entire duration of perfusion. Panel M: Tissue ATP levels (nmol/gram) over during 6 hours of perfusion. Figures at time 2-hour and 6-hour were normalised for the concentration at  $t = 0$  and expressed as fold increase. Levels of significance:  $*p < 0.05$  (Wilcoxon signed-rank test).

### 3.4.4 Liver function assessment

The lactate levels and clearance dynamics differed between the groups. In the NMP group, lactate levels peaked after 60 minutes, reached the viability criteria within 150 minutes and subsequently remained low. In the HOPE+NMP livers, it did not change through the hypothermic phase, and slowly increased in the rewarming phase with a subsequent rapid clearance just after 2 hours of NMP. The end-perfusion lactate levels were similar in viable livers from the NMP group and the HOPE+NMP group (1.6 mmol/L vs. 0.8 mmol/L,  $p = 0.17$ ). In the non-viable livers, lactate levels peaked after 60 minutes with minimum levels achieved by the end of the perfusion of 8.5 mmol/L. Three livers (60%) in the NMP group and 5 (100%)

in the HOPE+NMP were deemed viable ( $p = 0.22$ ). Details of the achievement of viability parameters are presented in Table 3.3. The two non-viable livers from the NMP group had the highest DRI, however this difference was not statistically significant (non-viable DRI 2.8 [2.5–3.2] vs. viable DRI 2.2 [1.9–2.2],  $p = 0.13$ ).

The glucose levels slowly increased within the initial 120 minutes of perfusion in the NMP group, followed by slow clearance until the end of the perfusion. In the HOPE+NMP group, they slowly increased through the hypothermic perfusion, followed by a drop (related to the perfusate fluid replacement) with a subsequent slow increase, and then decreased, reaching similar figures to the NMP group at the end of the perfusion (26 [20–40] vs. 16 [15–31] mmol/L,  $p = 0.22$ ).

Bile production at 6 hours of the perfusion was similar between both groups (NMP 7.39 mL/kg liver vs. HOPE+NMP 5.16 mL/kg liver,  $p = 0.82$ ). Bile pH (HOPE+NMP 7.27 [7.19–7.36] vs. NMP 7.26 [7.20–7.45],  $p > 0.99$ ) and bile glucose (HOPE+NMP 13 [9–16] vs. NMP 21 [14–27],  $p = 0.40$ ) were comparable between the groups at the end of the perfusion. Data is represented in Figure 3.2.

**Table 3.3: Viability criteria achievement by the livers in each group**

<b>Criteria</b>	<b>NMP (<i>n</i> = 5)</b>	<b>HOPE+NMP (<i>n</i> = 5)</b>
Lactate clearance ( $\leq 2.5$ mmol/L)	3 (60%)	5 (100%)
pH > 7.3 perfusate	2 (40%)	2 (40%)
Glucose metabolism	3 (60%)	4 (80%)
HA flow (> 150 mL/min)	5 (100%)	5 (100%)
PV flow (> 500 mL/min)	5 (100%)	5 (100%)
Homogeneous perfusion/ soft parenchyma	5 (100%)	5 (100%)
Bile production	3 (60%)	3 (60%)
<b>Viable liver</b>	<b>3 (60%)</b>	<b>5 (100%)</b>

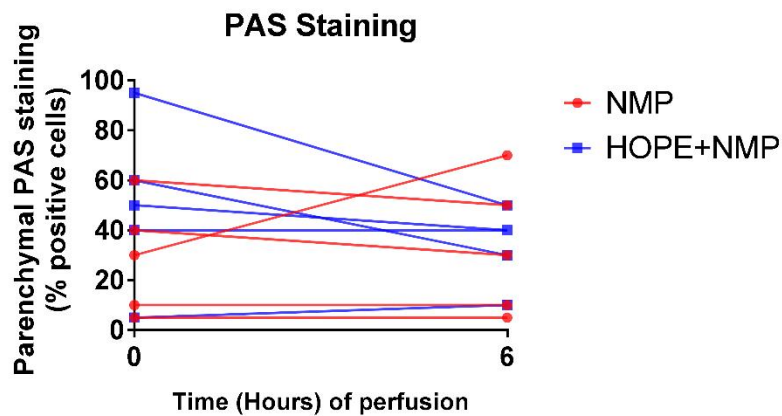
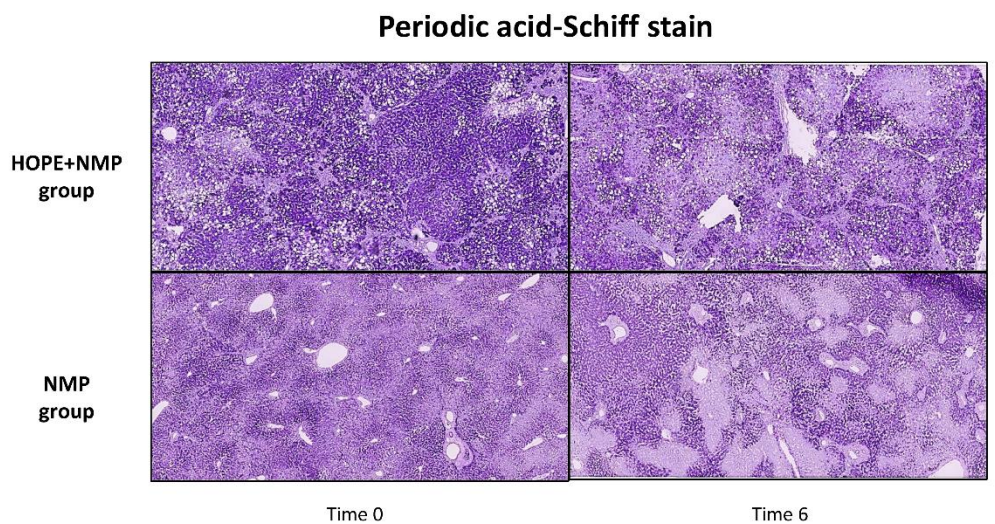
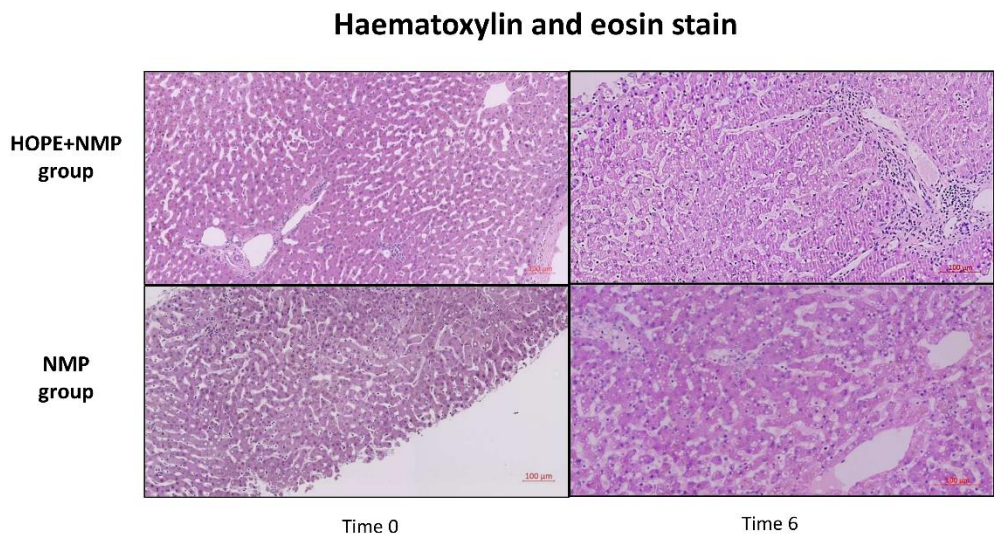
**Abbreviations:** NMP: normothermic machine perfusion of the liver; HOPE: hypothermic oxygenated perfusion of the liver; HA: hepatic artery; PV: portal vein

### 3.4.5 Histological assessment of the livers

Routine histological assessment showed no observable differences between the groups on the biopsy prior to the perfusion. At the end of the perfusion none of the livers presented with areas of parenchymal necrosis, congestion or cytoplasmic vacuolization. The median large droplet macrovesicular steatosis percentage was not significantly different between the groups (NMP 0% [0–30] vs. HOPE+NMP 10% [1–22],  $p = 0.98$ ) and only one liver from each group presented with steatohepatitis. The levels of hepatocyte glycogen depletion from start to end of the perfusion were similar in both groups [NMP 0% (–20/+10) vs. HOPE+NMP +10% (+2.5/+32.5),  $p = 0.22$ ] with a positive value reflecting an increase in the percentage of hepatocytes depleted of glycogen. Data are presented in Figure 3.3.

**Figure 3.3: Liver histology before and after machine perfusion.**

**Histology**



**Figure 3.3: Liver histology before and after machine perfusion.** The upper panel of images shows haematoxylin and eosin and the lower periodic acid-Schiff (PAS) staining at the beginning ( $t = 0$ ) and end of the perfusion ( $t = 6$  hours). No observable histological differences were seen between the two groups prior to or at the end of perfusion. There was no evidence of perfusion related ischaemic coagulative necrosis developed in any liver during perfusion. The percentage of areas of glycogen deposition (parenchymal PAS staining) from start to end of the perfusion was also similar in both groups.

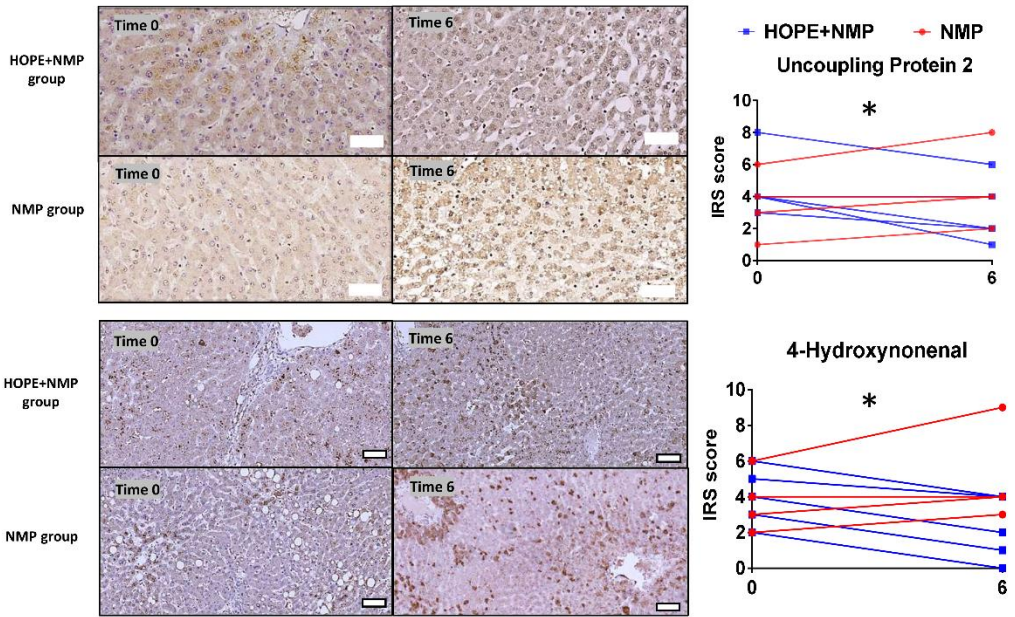
### 3.4.6 Immunohistochemical assessment of oxidative stress and tissue inflammation

Immunohistochemical analysis showed that livers subjected to NMP alone expressed higher levels of tissue markers of oxidative injury and inflammation compared to those subjected to HOPE+NMP by the end of the perfusion run. Livers which had HOPE showed a significant reduction in expression of markers associated with mitochondrial ROS production (uncoupling protein 2; NMP  $\Delta$ IRS: +1 vs. HOPE+NMP  $\Delta$ IRS: -2,  $p = 0.02$ ) and oxidative stress-mediated cellular injury (4HNE) expression reflecting lipid peroxidation (NMP  $\Delta$ IRS: +1 vs. HOPE+NMP  $\Delta$ IRS: -2,  $p = 0.008$ ). Both proteins were present predominantly in hepatocytes. Similarly, they showed decreased expression of CD14 at the end of the perfusion compared with commencement. A statistically significant opposite trend was seen for the livers perfused using NMP only (NMP  $\Delta$ IRS: +3 vs. HOPE+NMP  $\Delta$ IRS: -2,  $p = 0.008$ ). The intensity of the tissue inflammatory response was shown by different patterns of non-parenchymal cell staining for CD11b which was present on polymorphonuclear leukocytes (neutrophils and monocytes/macrophages). There was a significant difference in its level of expression between the two groups throughout the perfusion (NMP  $\Delta$ IRS: +3 vs. HOPE+NMP  $\Delta$ IRS: -1,  $p = 0.02$ ), suggesting a potential beneficial effect of the combined protocol. Intrahepatic endothelial cells play a key role in reperfusion injury by promoting leukocyte adhesion and retention. To evaluate the (cytokine-mediated) activation of endothelial cells, the percentages positive areas of VCAM-1 staining were determined. VCAM-1 expression decreased in both groups, however this was more pronounced in livers that had undergone HOPE (NMP  $\Delta\%$ VCAM-1: -0.5 vs. HOPE+NMP  $\Delta\%$ VCAM-1: -2.2,  $p = 0.05$ ). Data are presented in Figure 3.4.

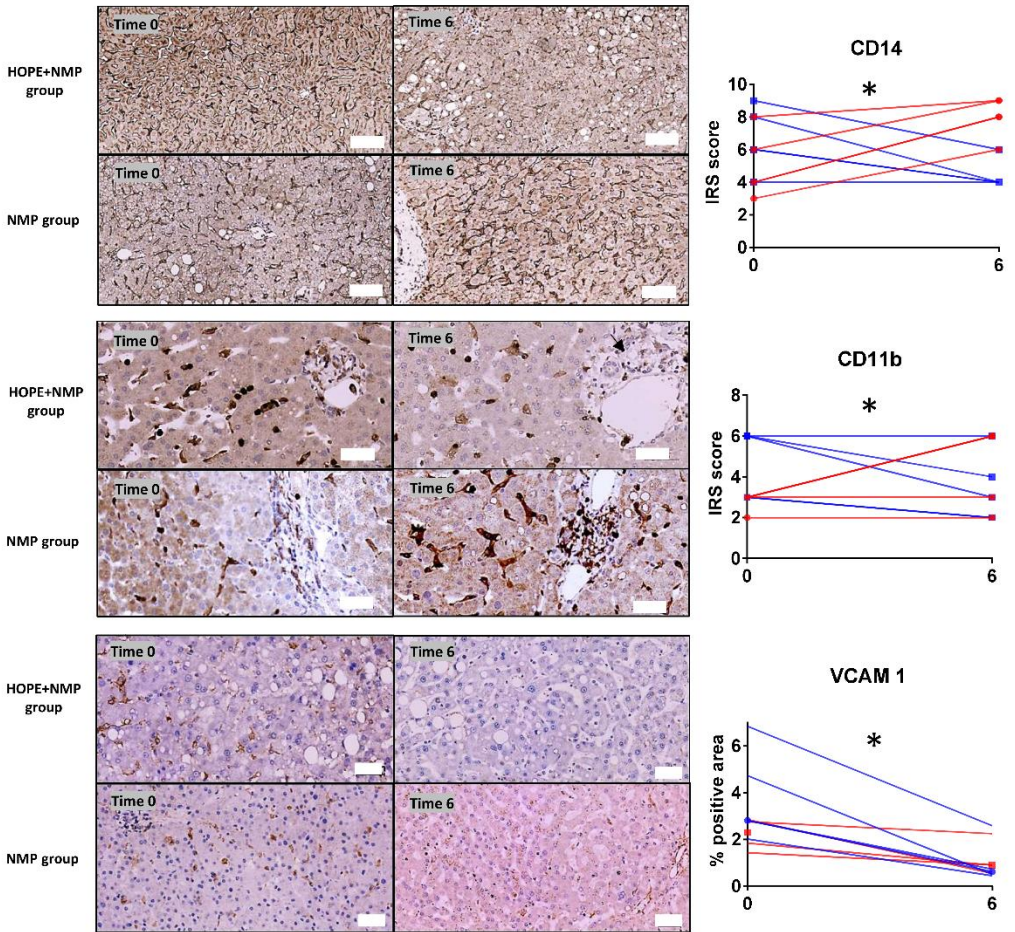


**Figure 3.4: Immunohistochemical analysis for markers of reactive oxygen species mediated damage and tissue inflammation.**

**(A) Immunohistochemistry parameters for reactive oxygen species production and associated damage**



**(B) Immunohistochemistry parameters for activation of the inflammatory cascade**



**Figure 3.4: Immunohistochemical analysis for markers of reactive oxygen species mediated damage and tissue inflammation.** Upper row of each panel shows representative images for the HOPE+NMP group and the lower images for the NMP group respectively prior to and at the end of perfusion (t = 0 and t = 6 hours). Section A shows there was a consistent reduction in expression of markers for oxidative injury in the HOPE+NMP group as reflected by the immunoreactive score (IRS). Contrastingly an opposite trend was seen for the NMP livers over the course of the perfusion. (B) Markers indicative of activation of the inflammatory cascade also showing reduced expression by the end of perfusion in the HOPE+NMP group. Whilst VCAM 1 expression decreased in both groups it trended towards being more pronounced in livers that had undergone HOPE+NMP. Bold outside border white squares at the bottom of figures represents 50  $\mu\text{m}$  and no border white squares 100  $\mu\text{m}$  scaling respectively. Levels of significance: \* $p < 0.05$  (Wilcoxon signed-rank test).

### 3.5 Discussion

Machine perfusion was developed to minimise damage during organ preservation and early clinical experience proved its superiority over SCS and positive impact on extended criteria liver utilisation (8, 12, 13, 22, 23). The two leading perfusion strategies, hypothermic and normothermic technique, have previously been viewed as diverse, or even competing, approaches each having advantages and disadvantages (24). In the present study, we compared a protocol which combined the two methods to evaluate whether the combination would provide the potential benefits of both. We found that initial HOPE perfusion promoted recovery of mitochondrial function, increased ATP energy stores and lowered tissue injury during subsequent NMP. Normothermic perfusion allowing viability assessment is seen by our group as an essential aspect of patient safety for transplantation of the highest-risk and / or currently unutilised livers. The combination of HOPE with NMP translated into the increased rate of functional recovery of livers pre-treated with HOPE compared to livers subjected to NMP alone.

Our team became an early adopter of the NMP technique, and we observed that a proportion of livers failed to recover their function (12). Those poor-quality organs, often exposed to prolonged cold ischaemia times, were unable to maintain vascular flows, clear lactate or produce any bile. With the mounting evidence of the protective mechanism of HOPE, we

designed the combined perfusion to assess if this intervention would yield a superior functional recovery for the highest-risk discarded livers (7, 22, 24, 25). Our key objective in designing this experiment was to imitate the real-life situation where clinical decisions are required regarding transplantability of a clearly suboptimal liver upon its arrival to the transplant centre.

Whilst the use of HOPE with subsequent NMP was reported by the Groningen group previously, their experiment was aimed at assessing benefit of HOPE compared to SCS and used the NMP phase to simulate in vivo reperfusion (26). In this scenario, the overall perfusion time on the device at the point of the functional assessment was beyond the certified scope of 6-hour use, limiting its value for translation towards clinical adoption (26, 27).

Our study represents a novel approach and shows unique data, being the first observations on human donor livers with viability assessment benchmarked by clinically used criteria (12, 28, 29). Previous rodent models of ECD livers showed that a short period of HOPE was able to promote recovery of mitochondrial function, optimising oxygen utilisation and recovering cellular ATP energy stores (30, 31). HOPE was suggested to promote metabolism of the succinate accumulated during the ischaemic period under hypothermic conditions, subsequently diminishing the reversal flow of electron from the mitochondria and the production of ROS during the rewarming (32).

Although there has been extensive mechanistic research using animal models, showing a downregulation in the tissue inflammatory responses, including activation of endothelial and resident immune cells of the liver, the evidence from human livers, especially those from the highest-risk donor pool, is very scarce (19, 24). In order to assess the extent of the injury developed during rewarming on the machine, we analysed numerous markers of non-parenchymal cell activation. In accordance with previous observations, we confirmed the

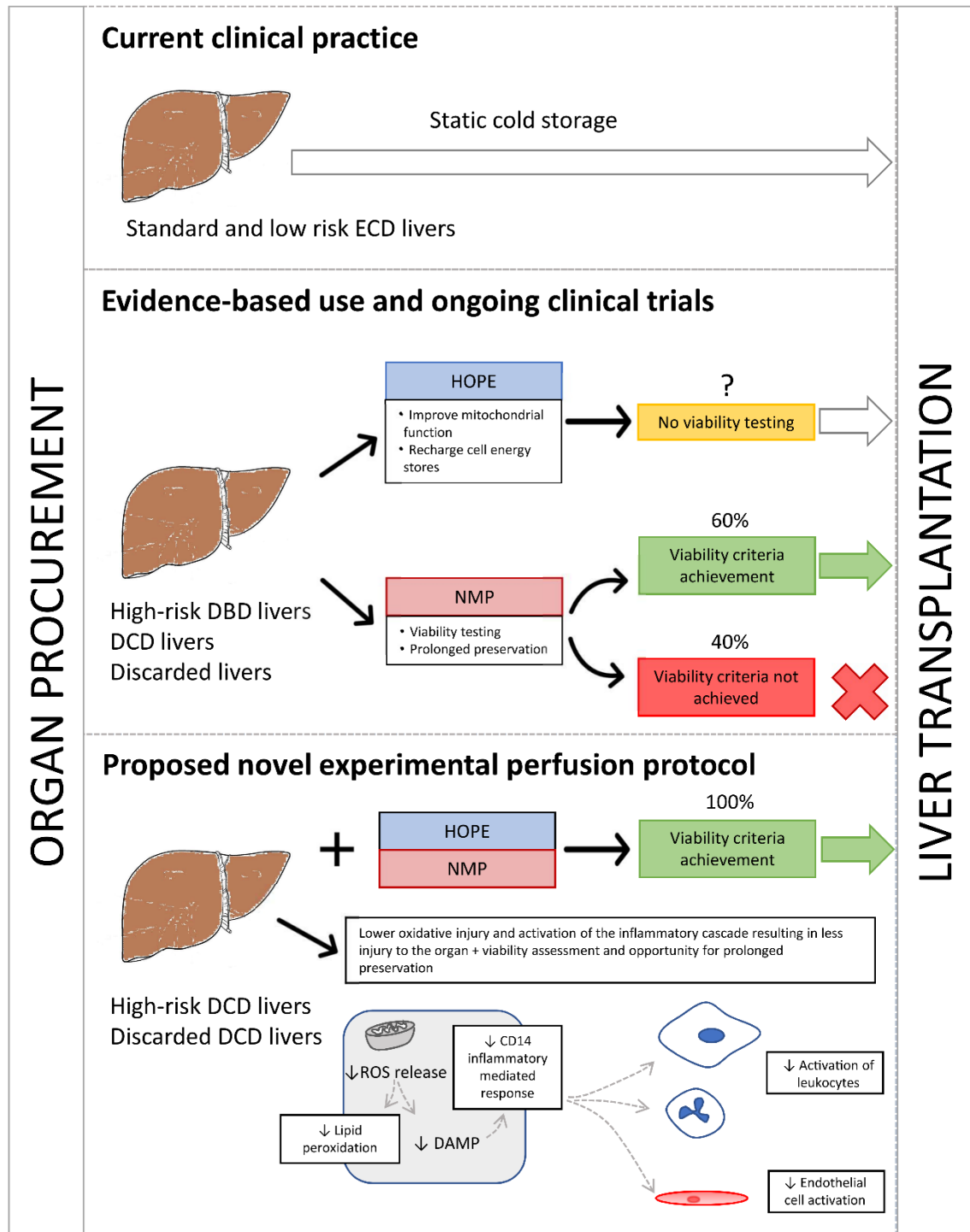


putative benefits of HOPE, in comparison to negligible changes or even an upregulation of detrimental processes observed during NMP perfusion alone (24, 32). We have shown changes in the mitochondrial respiratory rate during the HOPE phase of the combined perfusion group that correlated with lower activation of the inflammatory cascade and oxidative injury at the end of 6-hours of machine perfusion in comparison with NMP alone. Replacement of the perfusate, which may reduce toxic metabolites and detritus accumulated during the SCS and recirculating perfusate may also further enhance reduction of the tissue inflammatory response and injury in the HOPE+NMP group. The lack of the liver flush following the HOPE phase might alter the perfusion fluid constitution for the normothermic perfusion in the HOPE+NMP group; however, analysis of these minimal differences between the groups were beyond the scope of the present study (33, 34).

NMP provides a near-physiological environment for the liver by supplying oxygen and nutrients at normothermic condition, enabling the organ functional assessment. Our previous work on end-ischaemic NMP of declined human livers allowed us to define the viability criteria employed for transplantation of discarded human livers (12, 28). Setting an endpoint of achieving the objective criteria already used in our unit for clinical transplantation is an important strength of this study, though we appreciate there does not yet exist a widely accepted consensus regarding parameters for organ viability assessment (17, 26, 35, 36). Lactate clearance to levels lower than 2.5 mmol/L is a principal parameter for viability criteria used at our unit. Whilst this marker is seen in our clinical practice as highly sensitive and specific parameter to predict early graft failure due to primary non-function, Watson *et al.* recently advocated transaminase levels in the perfusate and bile quality assessment, by the pH and glucose content, to be superior in terms of predicting late graft loss due to biliary complications (36). This is a pertinent point, as the previous clinical studies have demonstrated the key

advantage of HOPE perfusion being prevention of non-anastomotic biliary strictures in DCD livers, and these organs might benefit most from the combined protocol (7, 37). The presented data showed improved viability also in DBD livers. Despite the limited number of perfusions included in our study, all assays from the HOPE+NMP group consistently showed cytoprotective effects and superior parameters, including clinical viability criteria (a diagrammatic summary is presented in Figure 3.5). This was demonstrated by all HOPE+NMP organs achieving the viability criteria, whilst 40% (2/5) of organs perfused by NMP alone failed to recover enough function to be deemed transplantable.

**Figure 3.5: Diagrammatic summary of the findings and proposal for the use of a combined protocol of *ex situ* machine perfusion encompassing hypothermic oxygenated perfusion (HOPE) and normothermic machine perfusion (NMP) for the recovery of extended criteria donor livers.**



**Figure 3.5: Diagrammatic summary of the findings and proposal for the use of a combined protocol of *ex situ* machine perfusion encompassing hypothermic oxygenated perfusion (HOPE) and normothermic machine perfusion (NMP) for the recovery of extended criteria donor livers.**

The top panel of the diagram illustrates current clinical practice for standard or low-risk extended criteria organs which are preserved using the traditional static cold storage. The middle panel shows the potentially beneficial effects of each machine perfusion protocol. HOPE perfusion does not permit objective organ viability assessment, which limits its potential use for high-risk extended criteria donor livers. NMP does allow viability assessment prior to transplantation making this option extremely valuable for viability assessment of high-risk extended criteria albeit resulting in less ECD livers achieving transplantable criteria than the combined protocol described here. Finally, the bottom panel summarises the outcome and possible mechanistic benefits of the combined protocol studied importantly showing the cumulative benefits of combining both cold and warm perfusion. This is reflected by demonstrating a higher rescue of high-risk ECD livers in the HOPE+NMP group than NMP alone.

The use of an acellular haemoglobin-based oxygen carrier was shown by our group and others to be a suitable alternative to the use of packed red cells in the context of NMP (16, 38). An acellular oxygen carrier-based fluid could be potentially used throughout the whole perfusion, avoiding any change of perfusate. Nevertheless, in logistic terms, the disconnection of the liver from the perfusion circuit for about 20 minutes, necessary to exchange the device perfusate, seemingly did not cause any measurable harm to the organs temporarily placed on ice. HOPE perfusion via portal vein only was previously shown to provide sufficient oxygenation for the entire liver, including the extra-hepatic biliary tree, without any need for manipulation of the hepatic artery at this stage (7, 24, 39).

The feasibility of combining variant perfusion techniques has been suggested before, though the advantages, caveats and logistics aspects of different combinations are yet to be seen (40). The current study is the first to show a combined protocol of HOPE+NMP that might be easily adopted to clinical practice. A short-term HOPE phase would optimise mitochondrial oxidative function decreasing oxidative injury, downregulating inflammatory response with subsequent mitigation of the ischaemia reperfusion injury during NMP. Those factors together might be essential to minimise any damage and improve functional recovery in the highest-risk ECD livers damaged by cold ischaemia during the SCS prior to the end-ischaemic machine perfusion.

This approach differs from NMP preservation when the organs are exposed only to a very short period of ischaemia (23). The Liver Assist device enables variation of perfusion strategies in temperatures ranging from 10 °C to 37 °C within the device-certified 6-hour timeframe. The presented study used 2 hours of HOPE and 4 hours of NMP, durations previously recommended by other groups, allowing use of a single perfusion kit and rendering the combined perfusion cost-effective (23, 26).

The present study has some caveats. It is not a transplant model, as this would not be clinically possible in the UK at this early investigative stage. Whilst the reperfusion injury could be simulated by a subsequent NMP with whole blood, we opted to study the organ functional recovery only, and have applied our current viability criteria to define its potential transplantability. Those criteria have been used by our team in clinical trials to safely transplant discarded human livers, allowing us to compare the findings and predict transplantability and clinical relevance of our pre-clinical proof of concept studies (12, 29). The use of discarded human livers for research confers the advantage of eliminating between-species variability, which is a limitation for animal models where the extrapolation of results to humans is questionable. The scarcity and intrinsic heterogeneity amongst discarded human livers, however, makes it difficult to achieve perfectly matched study groups.

Using an acellular oxygen carrier fluid also precludes any direct comparison of our data with frequently published machine perfusion studies' endpoints, including perfusate transaminases (23, 35, 36). The free haemoglobin concentration in the Hemopure<sup>®</sup>-based perfusate exceeded the maximum haemolytic index tolerance for our hospital clinical laboratory assessment, and we were unable to measure those subsequently by alternative methods from re-thawed frozen samples. As a similar perfusion fluid has already been used for clinical perfusions, however, we expect this might be soon widely adopted and our results validated by others.

In conclusion, this proof of concept study demonstrated that the combination of sequential HOPE and NMP is not only feasible, but that it may potentially improve the functional recovery of high-risk ECD livers compared with NMP alone. Whilst we do not suggest that this is an optimised protocol, this novel approach might be particularly beneficial for DCD organs. Further studies are needed to explore whether the combined protocol confers other benefits, such as the reduced biliary complications of HOPE or the safety of prolonged perfusions of NMP.

## **3.6 Appendices**

### **3.6.1 Complementary Methods**

#### **3.6.1.1 Immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded sections using the primary antibodies described below. Specie specific ImmPRESS™ (Peroxidase) Excel Amplified HRP Polymer Staining Kit detection was used for the staining.

- anti-uncoupling protein 2 (UCP2) antibody (ab203244 – Abcam, Cambridge, UK) rabbit polyclonal at 1/600 dilution.
- anti-4-hydroxynonenal (4-HNE) antibody (ab46545 – Abcam, Cambridge, UK) rabbit polyclonal at 1/200 dilution.
- anti-cluster of differentiation 14 (CD14) antibody (ab36595 – Abcam, Cambridge, UK) mouse monoclonal at 1/500 dilution.
- anti-vascular cell adhesion molecule 1 (VCAM-1) antibody (SAB1406579 - Sigma-Aldrich Inc, St Louis, MO, USA) mouse polyclonal at 1/400 dilution.
- anti-cluster of differentiation 11b (CD11b) antibody (ab52478 – Abcam, Cambridge, UK) rabbit monoclonal at 1/200 dilution.

All sections were wholly digitalized using the Axio Scan.Z1. Four areas at a magnification of 400x were randomly selected using the ZEN image analysis software. Areas of tissue edge were not included due to artefacts that could confound the analysis. Thereafter, the four random images were analysed for overall expression of the staining.

#### **3.6.1.2 Adenosine triphosphate quantification**

Measurement of adenosine triphosphate (ATP) levels was done accordingly with the following steps: 100 mg of frozen liver tissue was immediately homogenised in 1 mL SONOP Buffer

(0.372g EDTA in 130 mL ddH<sub>2</sub>O [adjusted to pH 10.9 with NaOH] = 370 mL of 96% Ethanol) using the GentleMacs system. Particulates were removed by centrifugation at 13,000xg. The protein concentration was determined in the supernatant with the use of a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, Illinois, USA) and the concentration adjusted to 300 µg/mL protein with the SONOP buffer. Samples were then diluted 10-fold in 100 µM phosphate buffer and ATP concentration determined using the ATP Bioluminescent Kit (FL-AA - Sigma-Aldrich Inc, St Louis, MO, USA). Concentrations were determined from a calibration curve on the same plate, corrected for amount of protein and expressed as nmol/g protein.

#### **3.6.1.3. Oxygen content in the perfusate equation**

$O_2 \text{ content} = (pO_2 \times K) + (sO_2 \times Hb \times c)$ ,  $pO_2$  in kPa,  $K$  equals 0.027 for  $O_2$  in water at 37 °C,  $Hb$  in mmol/L and  $c$  equals 91.12 mL $O_2$ /mmol for the oxygen binding capacity of haemoglobin.



### 3.6.2 Supplementary Table S3.1

**Table S3.1: Normothermic machine perfusion fluid constitution**

1000 mL (4 units) acellular oxygen haemoglobin carrier Hemopure® (haemoglobin glutamer-250-bovine; HBOC-201, Hemoglobin Oxygen Therapeutics LLC, Cambridge, MA)
1000 mL 5% w/v human albumin solution (Alburex 5, CSL Behring GmbH, Germany)
10,000 IU heparin (Wockhardt, UK)
30 mL sodium bicarbonate 8.4% (B. Braun Medical Limited, UK)
10 mL calcium gluconate 10%
500 mg vancomycin (Wockhardt, UK)
60 mg gentamicin (Cidomycin, Sanofi, UK)
50 mL 10% v/v Aminoplasma (B. Braun Medical Limited, UK)
0.2 mL Cernevit (Baxter Healthcare Ltd., UK)
0.1 mg phytomenadione (Konakion, Roche Products Ltd, UK)
Epoprostenol (Flolan, GlaxoSmithKline, UK, 2 µg/mL) continuous infusion commencing at 4 mL/hour

**Abbreviations:** MA- Massachusetts; UK- United Kingdom.

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## Chapter 4

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# THE USE OF AN ACELLULAR HAEMOGLOBIN-BASED OXYGEN CARRIER PERFUSATE FOR COLD-TO-WARM MACHINE PERFUSION OF THE LIVER: A FEASIBILITY STUDY

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### Manuscript in submission

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### *Transpl Int*

Y.L.B. designed the experiment, performed the experiment, interpreted the data and wrote the manuscript. R.W.L. assisted in some of the experiments. A.Sc. assisted in some of the experiments and in the analysis of the data. L.W. assisted with the technicalities of the laboratory tests. A.Sm. provided the pharmacological support for the experiments. J.A. assisted in some of the experiments. R.H.B. assisted in editing the manuscript. G.R. performed the histopathological assessment of the samples. M.T.PR.P., P.M. and D.F.M. offered surgical support for the experiments. H.M. designed the experiments and offered surgical support for the experiments. S.C.A. designed the experiments, assisted in the interpretation of the data and editing the manuscript.

## 4.1 Abstract

**Background:** *Ex situ* machine perfusion improves the metabolic parameters of donor livers, with reports of both hypothermic and normothermic approaches being used. However, combining both techniques may derive individual benefits. We investigated whether the use of an acellular haemoglobin-based oxygen carrier (HBOC)-based perfusate may facilitate this combination, the cold-to-warm machine perfusion. **Methods:** Ten discarded human donor livers were perfused for a total of 6 hours. Five in each group had: (1) 2 hours of hypothermic oxygenated perfusion (HOPE) using an oxygen carrier-free perfusate; followed by perfusate exchange and NMP with an HBOC-based perfusate; or (2) 2 hours of dual-hypothermic machine perfusion (D-HOPE) with sequential controlled oxygenated rewarming (COR) for 1 hour and then NMP using the HBOC-based perfusate uninterruptedly throughout the perfusion (cold-to-warm group). Markers of liver function and tissue injury were investigated and compared between groups. **Results:** Donor characteristics were comparable. Methaemoglobin levels were constant during D-HOPE and increased during NMP similarly to the HOPE+NMP group, whereas it did not affect tissue oxygen delivery. Hypothermic perfusion phase downregulated mitochondrial respiration and increased adenosine triphosphate levels in both groups. The cold-to-warm group presented higher arterial vascular resistance during rewarming/NMP (0.2 [0.2–0.3] vs. 0.1 [0.1–0.2] mmHg/mL/min/kg,  $p = 0.03$ ) with lower arterial flows (204 [155–230] vs. 318 [200–373] mL/min,  $p = 0.09$ ). At the end of NMP tissue expression of markers of reactive oxygen species production (uncoupling protein-2), oxidative injury (4-Hydroxynonenal, CD14) and inflammation (CD11b, VCAM-1) were comparable between the groups. **Conclusion:** This study suggests that despite changes in vascular dynamics, the use of an HBOC-based perfusate uninterruptedly from cold to warm is feasible and not harmful to extended criteria human donor livers. Its utilisation facilitates combination

of hypothermic and normothermic perfusions, offering the possibility of controlled rewarming of the organ.

## **4.2 Introduction**

*Ex situ* machine perfusion (MP) demonstrates superior preservation for extended criteria donor (ECD) livers compared to static cold storage and thus represents an option to safely expand their utilisation (1–3). The MP field has largely focused on oxygenated hypothermic MP (HMP) and normothermic MP (NMP) to date.

Briefly, NMP provides full metabolic support for donor organs which limits ischaemia and allows assessment of their metabolic function prior to transplantation (4, 5). Whilst viability assessment is fundamental to increase safely ECD livers utilisation, standardised objective protocols for their assessment are lacking (4–6). Alternatively, HMP performed either via portal vein (PV) only (hypothermic oxygenated machine perfusion [HOPE]) or via hepatic artery (HA) and PV (dual hypothermic oxygenated machine perfusion [D-HOPE]) was shown to enhance mitochondrial oxidative function, mitigating tissue injury during reperfusion and replenishing cellular adenosine triphosphate (ATP) stores (7–9).

Both MP techniques have reported benefits over standard static cold storage (3, 7, 8). There remains controversy as to whether markers of liver injury and function are comparable between HMP and NMP (5, 10). Our group has recently asserted that a combined protocol of HMP and NMP derives individual benefits of both techniques improving the metabolic parameters of ECD organs compared to NMP alone (11). In that study HOPE was performed using an oxygen carrier-free based perfusate, as at hypothermic temperatures, the supply of oxygen provided by diffusion is adequate to attend the reduced metabolic demand of the cells (9, 12); in addition, the use of red blood cells would not be possible owing to their risk of sludging at hypothermia (13). However, for the NMP phase, the use of an oxygen carrier is mandatory to fulfil the



metabolic demand of oxygen; and, therefore the perfusate was exchanged for an acellular, polymerised bovine haemoglobin-based oxygen carrier (HBOC) Hemopure<sup>®</sup> (hemoglobin glutamer-250 [bovine]; HBOC-201, Hemoglobin<sup>®</sup> Oxygen Therapeutics LLC, Cambridge, USA) based perfusate. Previous studies had reported the feasibility of using this compound in the setting of NMP and its advantages over red blood cells (14–16). Results showed that switching the perfusate from the HOPE to NMP phase in a combined perfusion protocol did not cause any harm to the liver. However, incorporating a single perfusate, compatible with perfusions over a range of temperatures, into the protocol would be a more feasible option as it would eliminate entirely any concerns of exposing the organ to additional unnecessary ischaemia time.

In the present study we hypothesised that with initial cannulation of the HA and PV, also known as the D-HOPE technique for the hypothermic phase, and with the use of a single HBOC-based perfusate throughout the entire perfusion, we would completely eliminate any concerns of additional unnecessary ischaemia time and optimise the logistics of a combined perfusion, thereby, increasing its clinical applicability. In addition, it would allow gradual rewarming of the organs (controlled oxygenated rewarming [COR]), which has also been suggested to mitigate reperfusion injury and improve graft function post-transplantation (17, 18). The aim of this study was to investigate the feasibility of a combined protocol of D-HOPE with sequential slow COR and NMP using a single HBOC-based perfusate during the perfusion process and assess its impact on the functional recovery of the organs.

### **4.3 Methods**

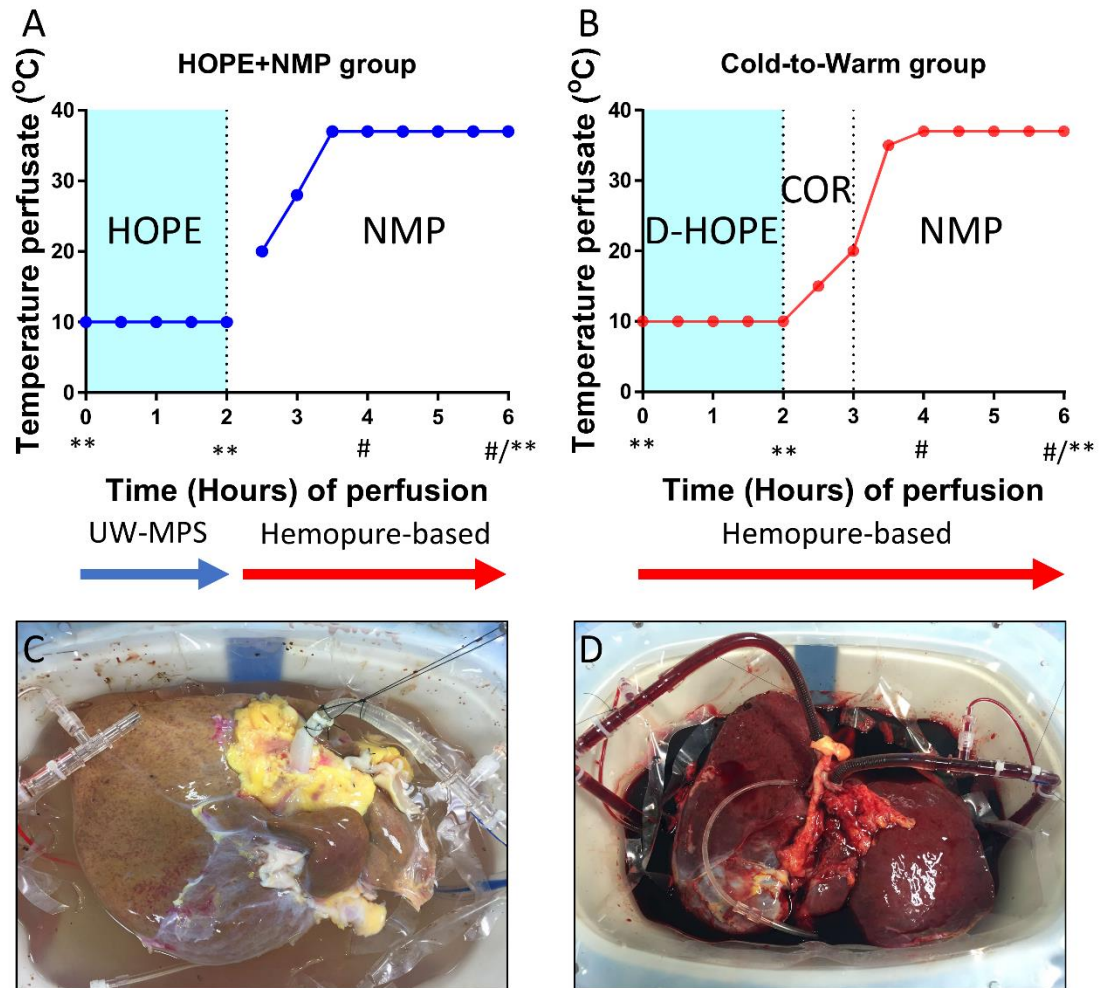
Ten discarded human livers were sequentially allocated to two experimental matched groups: (1) the HOPE+NMP group that had two hours of HOPE, using a Belzer MPS<sup>®</sup> UW Machine Perfusion Solution (UW-MPS) (Bridge to Life, London, UK) as the perfusate, followed by

NMP employing an HBOC-based perfusate [previously published group (11)]; and (2) the cold-to-warm group – this group had an HBOC-based perfusate since starting at time 0 that permitted simplifying changes to the protocol: HA and PV were cannulated at starting and D-HOPE was carried out for the hypothermic phase. Following this, the organs were gradually rewarmed (COR) and then had NMP for reperfusion and viability assessment. The total perfusion time was six hours for both groups. Perfusate, bile and tissue biopsies were sampled systematically. The detailed sampling protocol is presented in Figure 4.1.

#### **4.3.1 Source of discarded human livers**

All livers were retrieved with the intention of clinical transplantation according to the National Organ Retrieval Service standards. After the procurement, they were stored on ice in Belzer University of Wisconsin (UW<sup>®</sup>) Cold Storage Solution (Bridge to Life) and then rejected for transplantation by all transplant centres in the United Kingdom and offered for research. The study ethical approval was obtained by the London-Surrey Borders National Research Ethics Service and the NHSBT Ethics Committee (references 13/LO/1928 and 06/Q702/61, respectively).

**Figure 4.1: Study design.**



Donor human livers had standard procurement, they were cold flushed and stored. Once rejected for transplantation, they were offered for research and consecutively allocated to the two experimental groups. Image A shows the protocol for the HOPE+NMP group, livers had hypothermic oxygenated perfusion (HOPE) using Belzer MPS® UW Machine Perfusion Solution for 2 hours. HOPE was performed at 10 °C via portal vein only, as represented on Image C. After 2 hours the perfusate was changed to an acellular, haemoglobin-based oxygen carrier (HBOC) Hemopure® (HBOC-201, Hemoglobin® Oxygen Therapeutics LLC, Cambridge, USA) based perfusate for the rewarming and normothermic machine perfusion (NMP). The livers from the cold-to-warm group (Image B) were fully cannulated at the start of the perfusion, including portal vein, hepatic artery and common bile duct (Image D). They received an HBOC-based perfusate from time 0. For this group, the livers had 2 hours of dual hypothermic oxygenated perfusion (D-HOPE) at 10 °C followed by 1 hour of slow rewarming to 20 °C (controlled oxygenated rewarming [COR]) and then NMP. Menghini and wedge biopsies were collected at time 0, 2 and 6 hours (\*\*) and immediately fixed in formalin or snap-frozen in liquid nitrogen. Blood gas analysis was carried out and perfusate was sampled at 30 min time intervals throughout. In addition, bile was collected and weighted at time 4 and 6 hours (#).

### **4.3.2 Organ preparation and machine perfusion procedure**

After arrival at our centre the livers had a standard bench preparation as described elsewhere (19) followed by flushing with two litres of 5% glucose solution. The Liver Assist Device (Organ Assist, Groningen, Netherlands) was used for MP. The perfusate temperature and perfusion pressures were set by the operator as specified below. The measured flow rates and calculated resistances, as shown on the device's display in real time, were recorded every 30 minutes.

#### **4.3.2.1 Hypothermic oxygenated perfusion (HOPE)**

The HOPE phase was performed via PV only, using three litres of UW-MPS, with the temperature set at 10 °C. The target flow was 0.1 mL/min/g of liver with a maximum pressure of 3 mmHg. The target oxygen perfusate pressure (pO<sub>2</sub>) was 80–100 kPa. After 2 hours of HOPE, the perfusion was stopped, and the liver temporarily placed on ice. The system was then drained and subsequently refilled with the NMP perfusion solution. The HA and common bile duct were cannulated and the NMP perfusion commenced. The perfusion fluid exchange until the start of NMP took on average 20 minutes.

#### **4.3.2.2 Dual hypothermic oxygenated perfusion (D-HOPE)**

Before initiation of the perfusion the cystic duct was ligated, and the common bile duct cannulated with a 12 French biliary drain. The PV was cannulated with the designated cannula provided by the manufacturer and the coeliac trunk with a 16 French plastic tube. The D-HOPE perfusion was performed via PV and HA, using a perfusate consisted of 1000 mL (4 units) Hemopure® complemented with human albumin solution. Temperature was set at 10 °C and the target of flow on the PV was 0.1 mL/min/g of liver with a maximum pressure of 5 mmHg.

The pressure on the HA was 20–25 mmHg and oxygen offered to achieve a  $pO_2$  of 60 kPa. After two hours of D-HOPE, livers were slowly rewarmed using the COR technique.

#### **4.3.2.3 Controlled oxygenated rewarming (COR)**

This phase was sequential to the D-HOPE period and lasted for 1 hour. At the beginning PV pressure was increased to 5 mmHg and HA pressure to 30 mmHg. The temperature was raised 3 °C each 20 minutes during the period targeting 20 °C until the end of 1 hour. At the beginning of this phase the HBOC-based perfusate was supplemented with antibiotics and other complements (specified in Supplementary Table S4.1).

#### **4.3.2.4 Normothermic machine perfusion (NMP)**

The perfusion fluid for the NMP phase was Hemopure<sup>®</sup>-based as detailed in Supplementary Table S4.1. The UW-MPS was changed for the NMP perfusate before commencement of the NMP for the HOPE+NMP group. The cold-to-warm group already had this perfusate circulating in the system and, therefore, there was no change. Both groups had, at the start of the NMP phase, a perfusate temperature of 20 °C, pressures of 5 mmHg on the PV and 30 mmHg on the HA. From there, the temperature was raised incrementally to 37 °C. The target of flow was 0.25 mL/min/g liver tissue on the arterial side and 0.75 mL/min/g liver tissue on the venous circuit. To achieve these flows, perfusion pressures on the device were adjusted between 30–50 mmHg (mean pressure) on the arterial side and 8–10 mmHg on the PV.  $pO_2$  was set at 40 kPa in both groups at this phase.

#### **4.3.3 Assessment of liver metabolism**

Oxygen uptake was calculated during the HOPE perfusion (oxygen carrier-free based perfusate) as the the oxygen inflow minus the outflow in kPa, corrected by liver weight. For the HBOC-

based perfusate the oxygen consumption was calculated from the difference between the oxygen content after the oxygenator and return into the oxygenator in the venous circuit. Oxygen content was calculated as the sum of the free dissolved oxygen fraction to the haemoglobin-bound oxygen fraction (equation located in the supplementary methods) as described elsewhere (20).

The recovery of appropriate liver metabolism was assessed during NMP by our unit's published criteria. The major criterion was perfusate lactate levels < 2.5 mmol/L within 6 hours of perfusion, associated with the evidence of bile production, glucose consumption, stable vascular flows and homogeneous parenchymal perfusion (4, 21).

#### **4.3.4 Histopathological assessment of hepatocyte injury**

Liver biopsies obtained prior to and at the end of perfusion were immediately fixed in formalin, processed and embedded in paraffin. Thereafter, 4 µm sections were cut and stained with haematoxylin and eosin (H&E) as well as periodic acid-Schiff (PAS). H&E sections were semi-quantitatively graded for ischaemic-type coagulative necrosis, macrovesicular steatosis (large lipid droplets filling up the hepatocytes and displacing the nucleus to the periphery) and pre-existing liver disease. The PAS-stained sections were scored for the percentage of positive areas of glycogen, and the variation between the beginning and end of the perfusion was compared across groups. Histological assessment was blinded and conducted by an experienced liver transplant pathologist.

#### **4.3.5 Immunohistochemical assessment of oxidative stress and tissue inflammation**

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections to assess surrogate markers of oxidative injury and tissue inflammation. The uncoupling protein 2 (UCP2) is a mitochondrial inner membrane protein that uncouples the electron transport chain

from oxidative phosphorylation. Elevated UCP2 expression is associated with increased reactive oxygen species (ROS) production (22–24). Oxidative tissue injury was evaluated by the expression of the 4-hydroxynonenal (4-HNE), a product of lipid peroxidation in cells. For the assessment of tissue inflammation, we analysed the following markers: (1) cluster of differentiation (CD)14, a lipopolysaccharide receptor which is part of the toll-like receptor 4 signalosome. It is essential for the recognition of ligands such as damage-associated molecular patterns known to be upregulated during ischaemia-reperfusion injury (25–27); (2) CD11b is an integrin on the surface of leukocytes; upregulation of its expression indicates activation of the cells by substances, including ROS (28, 29); and (3) the vascular cell adhesion molecule 1 (VCAM-1), a cell adhesion molecule expressed in cytokine-activated endothelial cells that mediates leukocyte transendothelial migration. All primary antibodies were detected using specific ImmPRESS™ Excel Amplified HRP Polymer Staining kits (Vector Laboratories, Burlingame, CA, USA) specific to the respective mouse or rabbit immunoglobulin isotype. A list of primary antibodies and dilutions used is provided in the supplementary material.

Four images of each section, excluding the edges, were randomly selected for analysis (×400 magnification). A semiquantitative scoring system, the modified immunoreactive score (IRS) (30), was obtained by multiplying the score for intensity (0: no colour reaction; 1: mild reaction; 2: moderate reaction; 3: intense reaction) and distribution (0: no positive cells; 1: < 10% positive cells; 2: 10–50% positive cells; 3: 51–80% positive cells; 4: > 80% positive cells) to obtain a final score between 0 and 12. The change in overall tissue expression of staining ( $\Delta$ IRS) was determined by subtracting the IRS scores after 6 hours of perfusion from the score prior to perfusion: negative values indicated a decrease and positive values an increase in the expression of staining.

#### **4.3.6 Assessment of tissue adenosine triphosphate (ATP) levels**

Quantification of ATP levels was carried out by homogenisation of liver tissue. The concentration was determined with the ATP Bioluminescent Assay kit (FLAA, Sigma-Aldrich Inc, St Louis, USA). More details are provided in the supplementary material.

#### **4.3.7 Statistical analysis**

Continuous variables were expressed as median with interquartile range (IQR) and categorical variables as the absolute number with percentage frequencies. Comparisons between groups were performed via Fisher's exact test for categorical variables, the Mann–Whitney *U* test for independent continuous variables and the Wilcoxon signed-rank test for repeated measurements over time on the same sample. The statistical level of significance was set at  $p < 0.05$ . GraphPad Prism (version 6.04 for Windows, GraphPad Software, La Jolla, USA) software was employed for graph creation and all statistical analyses were performed on the Statistical Package for the Social Science version 22 software (IBM Corp, Armonk, NY).

### **4.4 Results**

#### **4.4.1 Donor demographics and organ characteristics**

The median donor age for all livers was 54 years (IQR: 51–55), donor body mass index 31 kg/m<sup>2</sup> (24–36) and donor risk index 2.1 (2.0–2.6). Median cold ischaemia time was 663 minutes (510–712) and warm ischaemia time for donors after circulatory death (DCD) 8 minutes (7–31). Detailed donor demographics and donor organ characteristics are presented in Table 4.1. Each experimental group had three DCD livers and the groups were comparable regarding donor and liver characteristics (Table 4.2).



**Table 4.1: Donor demographics, liver characteristics and machine perfusion parameters.**

Liver number	Cold-to-warm 1	Cold-to-warm 2	Cold-to-warm 3	Cold-to-warm 4	Cold-to-warm 5	HOPE+NMP 1	HOPE+NMP 2	HOPE+NMP 3	HOPE+NMP 4	HOPE+NMP 5
<b>Donor information</b>										
Age	73	51	55	57	52	54	50	54	38	55
Donor type	DBD	DCD	DBD	DCD	DCD	DCD	DBD	DBD	DCD	DCD
Sex	Male	Male	Male	Female	Female	Male	Female	Female	Male	Female
Height (cm)	164	189	172	160	160	179	158	170	183	160
Bodyweight (kg)	90	76	85	70	90	123	60	87	85	90
Body mass index (kg/m <sup>2</sup> )	33	21	29	27	35	38	24	30	25	35
US - Donor risk index	2.0	2.1	2.0	2.5	2.9	2.5	2.0	1.9	1.8	3.0
UK - donor liver index	1.4	1.8	1.1	1.9	2.1	1.8	1.1	1.0	1.8	1.9
ET - donor risk index	2.0	2.7	2.0	2.5	2.8	2.9	2.2	1.9	2.3	2.8
Peak ALT (IU/L)	33	74	72	135	81	189	14	271	37	741
Days on ventilator	4	3	2	11	4	5	2	2	4	4
Co-morbidities and risk history	HTN	Smoker	Diabetes (type 2)	Alcohol misuse	Smoker, HTN	Diabetes (type 2) HTN	Smoker, Alcohol misuse	Diabetes (type 1), Smoker	Active Smoker	HTN
Cause of death	HBD	HBD	ICH	ICH	HBD	HBD	ICH	ICH	ICH	HBD
<b>Liver characteristics</b>										
Liver weight (g)	1652	1900	1920	2140	1598	2600	1838	2060	1753	1935
Donor warm ischaemic time (minutes)	NA	8	NA	10	14	31	NA	NA	42	8
Cold ischaemia time (minutes)	532	645	793	430	934	497	682	491	660	510
Retrieval location	Regional	Extra-zonal	Extra-zonal	Regional	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal	Extra-zonal
Reason for clinical rejection	Donor cancer	Steatosis	Steatosis	Steatosis	Donor cancer	Poor flush	Steatosis	Steatosis	Poor flush	Steatosis, poor flush
Large droplets macrovesicular steatosis (paraffin sections)	0%	0%	20%	40%	0%	10%	0%	15%	3%	30%
<b>Machine perfusion parameters</b>										
Lactate (mmol/L)										
Highest	6.7	9.1	12.0	12.6	10.2	9.1	8.9	10.4	4.6	7.8
Lowest	0.3	0.7	5.1	2.4	0.2	0.6	1.1	1.8	0.2	0.8
At the end of 6-hour perfusion	0.3	0.7	6.3	2.4	0.2	0.6	1.1	1.8	0.2	0.8
Total Bile production (g)	5.0	3.2	0.0	10.0	0.0	57.0	0.0	0.0	15.9	24
Mean Arterial flow (mL/min)	92	102	132	77	127	292	299	234	152	398
Mean Portal vein flow (mL/min)	636	619	390	322	451	1412	1523	1602	1406	1582
Mean liver mass perfusion (mL/g/min)	0.4	0.4	0.3	0.2	0.4	0.7	1.0	0.9	0.9	1.0
Viability criteria met	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes

**Abbreviations:** HOPE: hypothermic oxygenated perfusion of the liver; NMP: Normothermic machine perfusion of the liver; DCD: donation after circulatory death; DBD: donation after brain death; US: United States; UK: United Kingdom; ET: Eurotransplant; ALT: alanine aminotransferase; HTN: hypertension; ICH: intracranial haemorrhage; HBD: hypoxic brain damage; NA: not applicable. **Note:** Donor warm ischaemic time was defined as the interval between the systolic blood pressure less than 50mmHg or/and arterial oxygen saturation to less than 70% to commencing the aortic cold perfusion in the donor.

**Table 4.2: Donor demographics, liver characteristics and machine perfusion data.**

	Cold-to-Warm ( <i>n</i> = 5)	HOPE+NMP ( <i>n</i> = 5)	<i>p</i> -value
<b>Donor information</b>			
Age, years	55 (52–57)	54 (46–54)	0.87
DCD livers	3 (60%)	3 (60%)	> 0.99
Sex, male	3 (60%)	2 (40%)	0.53
Height (cm)	164 (160–172)	179 (169–181)	0.11
Bodyweight (kg)	85 (76–90)	90 (87–106)	0.98
Body mass index (kg/m <sup>2</sup> )	29 (27–33)	35 (30–37)	0.73
Donor risk index	2.1 (2.0–2.5)	2.5 (2.2–2.7)	0.20
UK donor liver index	1.8 (1.2–2.0)	1.8 (1.0–1.8)	0.55
ET donor risk index	2.5 (2.0–2.8)	2.3 (2.0–2.8)	0.84
Peak ALT (IU/L)	74 (72–81)	189 (37–271)	0.18
Days on ventilator	4 (3–4)	4 (2–4)	0.45
<b>Liver characteristics</b>			
Liver weight (g)	1900 (1749–2020)	1935 (1838–2060)	0.58
Donor warm ischaemic time (minutes)	8 (6–11)	31 (19–36)	0.10
Cold ischaemia time (minutes), DCD	705 (537–789)	682 (586–708)	0.86
Cold ischaemia time (minutes), DBD	658 (523–793)	586 (490–682)	0.93
Macrovesicular steatosis (%)	0 (0–30)	10 (1–22)	0.97
<b>Machine perfusion parameters</b>			
Lactate (mmol/L)			
Highest	10.2 (9.1–12.0)	8.9 (7.8–9.1)	0.37
Lowest	0.7 (0.3–2.4)	0.8 (0.6–1.1)	0.45
Last	0.7 (0.3–2.4)	0.8 (0.6–1.1)	0.45
Total Bile production (g)	4 (0–6)	16 (0–40)	0.20

**Abbreviations:** NMP: normothermic machine perfusion of the liver; HOPE: hypothermic oxygenated perfusion of the liver; DCD: donation after circulatory death; UK: United Kingdom; ET: Eurotransplant; ALT: alanine aminotransferase; DBD: donation after brain death.

**Note:** Continuous variables presented as median and interquartile range, dichotomous variables as absolute number and percentage. Donor warm ischaemic time was defined as the interval between the systolic blood pressure less than 50mmHg or/and arterial oxygen saturation to less than 70% to commencing the aortic cold perfusion in the donor.

#### **4.4.2 Stability of an HBOC-based perfusate at variable temperatures**

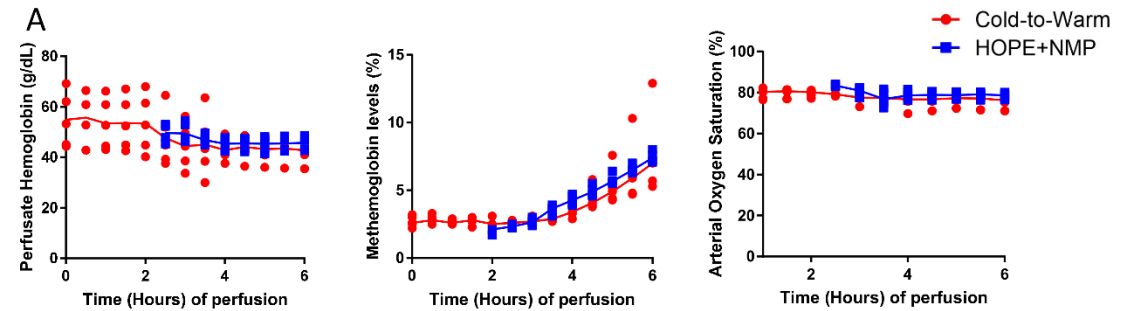
Haemoglobin levels of the HBOC-perfusate were comparable between the start and end of the hypothermic phase (D-HOPE) (53.2 [44.7–65.6] vs. 52.8 [42.5–64.7] g/dL,  $p = 0.59$ ). At the beginning of the COR phase, the supplements were added to the HBOC-based perfusate and a dilutional effect was observed between 2 and 3 hours. The median haemoglobin levels were similar at the end of NMP between the groups (HOPE+NMP 46.7 [42.9–47.7] vs. cold-to-warm 47.5 [38.2–50.4] g/dL). Methaemoglobin (MetHb) levels were stable during the D-HOPE and COR phases and then rose with the rewarming/NMP phase. Despite this trend the levels of oxygen saturation were constant. Details are found in Figure 4.2.

#### **4.4.3 The effectiveness of an HBOC-based perfusate on mitochondrial respiration and energy replenishing during the hypothermic phase**

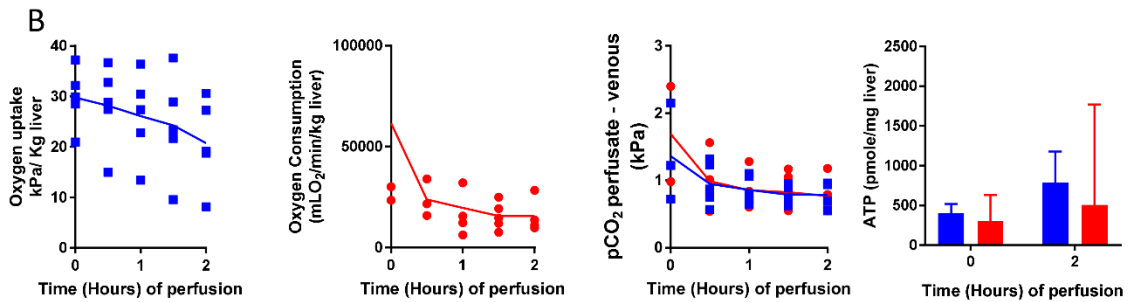
During the HOPE phase, the livers exhibited down regulation of mitochondrial respiration with a decrease in the oxygen uptake rate and CO<sub>2</sub> release in the perfusate. Similarly, during D-HOPE, the livers presented a fall in oxygen consumption rate from time 0 to 2 hours (26810 [23466–30154] vs. 12399 [11062–44705] mL O<sub>2</sub>/min/kg liver,  $p = 0.10$ ) with a drop in pCO<sub>2</sub> levels, reaching similar levels at 2 hours (HOPE+NMP 0.8 [0.6–1.0] vs. cold-to-warm 1.2 [1.0–1.7] kPa,  $p = 0.28$ ). Pre-perfusion ATP levels were comparable between groups ( $p > 0.99$ ). At the end of the 2 hours HMP, it increased 1.8-fold in the HOPE+NMP group and 2.5-fold in the cold-to-warm group. Details are provided in Figure 4.2.

**Figure 4.2: Stability of HBOC-201-based perfusate at different temperatures and its impact on mitochondrial function at hypothermic temperatures.**

Stability of a HBOC-201 (Hemopure®) based perfusate at wide range of temperatures



Mitochondria respiration and energy replenishing during the hypothermic phase

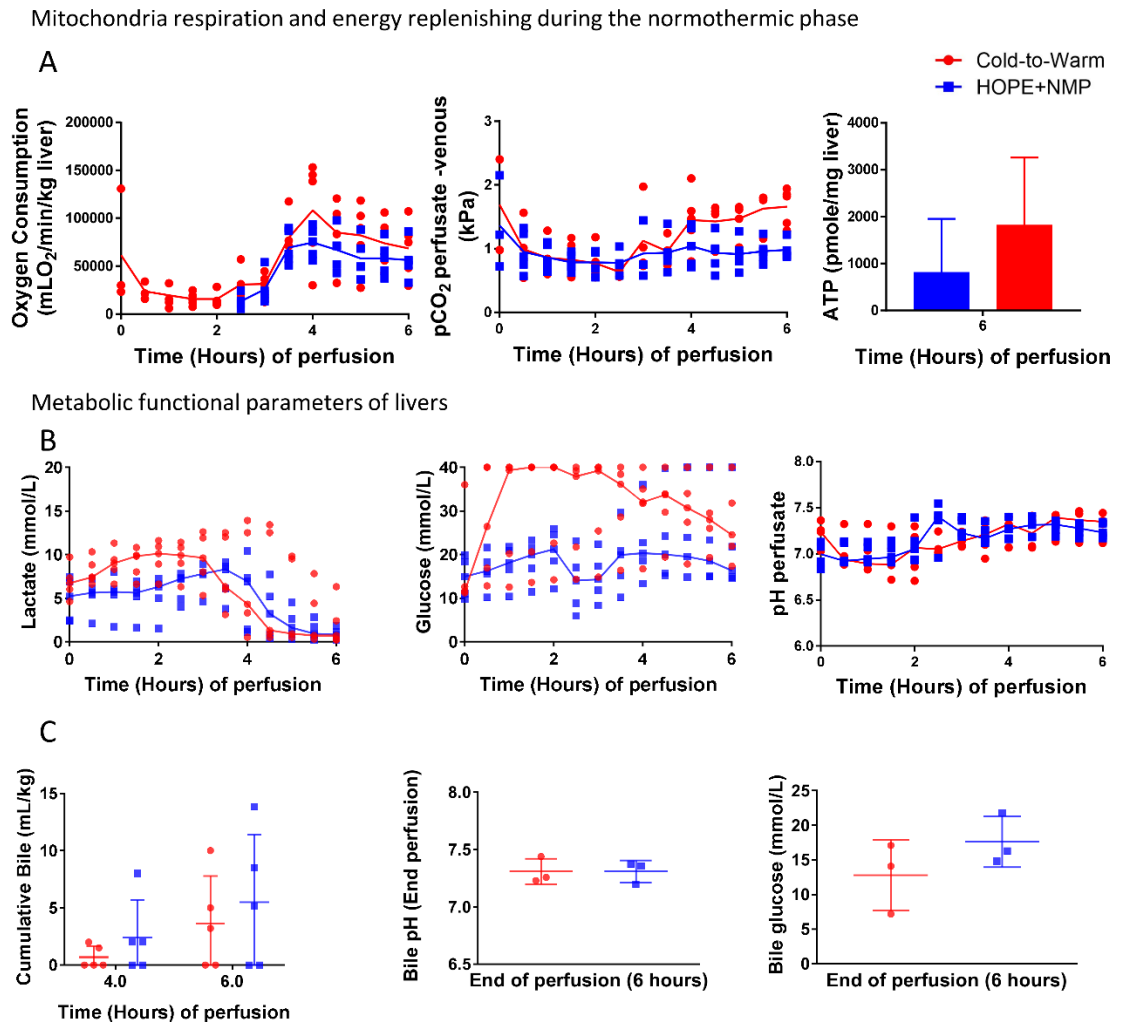


Panel A shows on the left-hand graph that haemoglobin levels were stable during the D-HOPE phase, and after rewarming, they were similar to the HOPE+NMP group that received fresh HBOC-based perfusate. Methaemoglobin levels (middle graph) were stable during D-HOPE and increased slowly during the rewarming, reaching comparable levels to the HOPE+NMP group during the normothermic phase. Despite the increase in the levels of methaemoglobin, arterial oxygen saturation was constant throughout the perfusion. Panel B compares markers of mitochondrial respiration between the study groups. Both groups presented a downward trend in oxygen requirements (oxygen uptake—HOPE+NMP group and oxygen consumption—cold-to-warm group), with a similar drop in the release of carbon dioxide (CO<sub>2</sub>) in the perfusate. Reflecting an efficient mitochondrial oxidative function, both groups replenished adenosine triphosphate (ATP) stores during the hypothermic phase. In the graphs, dots represent individual organs at the time points, and the line is the median of the values for each group. For the bar graph, the median and interquartile range are represented.

#### **4.4.4 The effect of hypothermic perfusion on the rewarming and the normothermic phase**

During the COR phase, oxygen consumption increased slightly and reached 40641 mL $O_2$ /min/kg liver (36576–44705). After rewarming, during the NMP phase, despite not being statistically significant, the peak of oxygen consumption was higher for the cold-to-warm group (138603 [52261–149192] vs. 75883 [59226–89717] mL $O_2$ /min/kg,  $p = 0.22$ ). The  $CO_2$  levels increased steadily for the cold-to-warm during the NMP phase and were stable for the HOPE+NMP group. At the end of the perfusion run, the levels of ATP increased 2.7-fold in the HOPE+NMP group and 5.8-fold in the cold-to-warm group (Figure 4.3).

**Figure 4.3: Mitochondrial and hepatic functional markers during the normothermic phase.**



Panel A shows a similar incremental rate of oxygen consumption during the rewarming period, which finally culminated in a higher peak at the beginning of the normothermic phase for the cold-to-warm group. The rate of carbon dioxide (CO<sub>2</sub>) release in the perfusate followed a similar trend to the oxygen consumption, and the adenosine triphosphate (ATP) levels reached higher figures at the end of the 6 hours of perfusion in the cold-to-warm group than with the HOPE+NMP. Panel B represents parameters of metabolic function of the organs during the perfusion. During the hypothermic phase, lactate levels increased slightly for the livers that had D-HOPE and were constant throughout HOPE. After rewarming, the lactate peak was comparable between groups, and then the lactate clearance was more effective in the cold-to-warm group resulting in similar levels at the end of the perfusion. There was evidence of gluconeogenesis at the beginning of the D-HOPE perfusion, and thereafter the organs start to consume glucose during the NMP phase. For the HOPE+NMP group, there was a sudden drop related to perfusate change at 2-hour perfusion and then the livers start to metabolise glucose during the NMP phase. Perfusate pH was similar between groups during the perfusion. Three livers in each group produced bile along the perfusion (Panel C). The bile pH was comparable between them, as was the glucose content. In the graphs, dots represent individual organs at the time points, and the line is the median of the values for each group. For the bar graph, the median and interquartile range are represented.

#### 4.4.5 Vascular flow dynamics

Median flow rate on the HA for the cold-to-warm at the beginning of the HMP phase was 42 mL/min (24–89) and it reached 54 mL/min (19–70) at the end of this phase. Those figures were similar during the COR phase (59 mL/min [55–118]). During the NMP phase, at 4 and 4.5 hours of perfusion, there was a trend of higher arterial flows on the HOPE+NMP group ( $p = 0.09$  and  $p = 0.06$ , respectively). As appropriate arterial flows (0.25 mL/g liver) were achieved the pressure were not further increased to the maximum of 50 mmHg, as it was done for the cold-to-warm group. Despite this adjustment, the perfusion pressures during NMP phase were similar between the experimental groups along the perfusion ( $p = 0.22$ ). At the end of the NMP the median flow rates were comparable between the groups (HOPE+NMP 365 [256–388] vs. cold-to-warm 310 [250–332] mL/min).

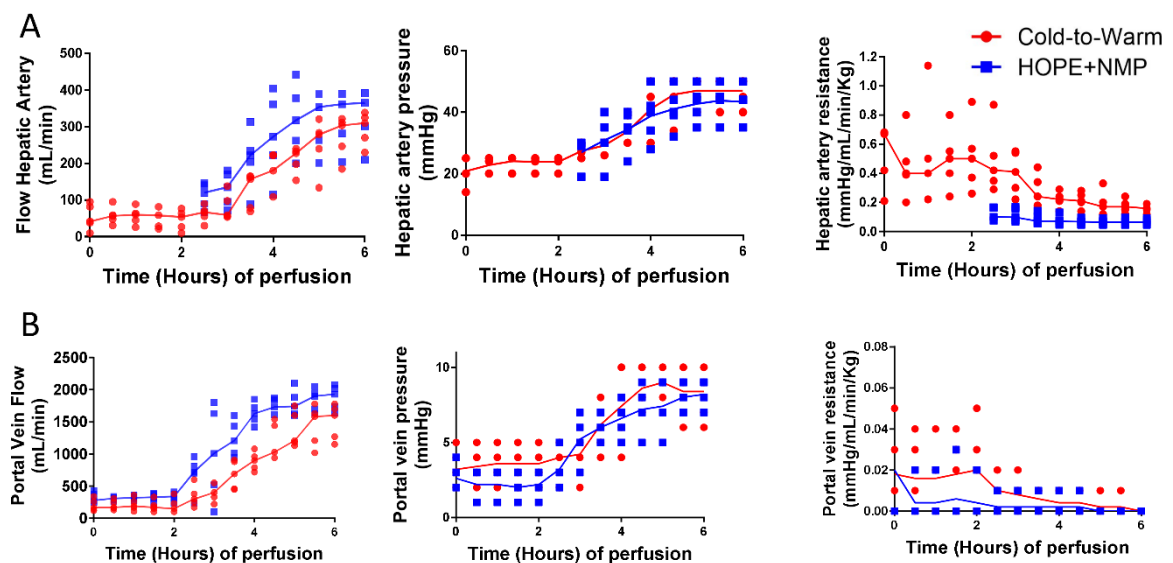
Median arterial vascular resistances were high during D-HOPE (0.3 mmHg/mL/min/kg) and decreased slightly during the COR phase (0.2 mmHg/mL/min/kg). When arterial perfusion was started in the HOPE+NMP group, HA vascular resistance was lower than in the cold-to-warm (0.2 [0.1–0.3] vs. 0.4 [0.3–0.7] mmHg/mL/min/kg,  $p = 0.03$ ). It remained significantly lower in the HOPE+NMP group until 5 hours of perfusion and then decreased for the cold-to-warm group, reaching a non-significant difference at the end of the normothermic phase (0.1 [0.1–0.2] vs. 0.2 [0.1–0.2] mmHg/mL/min/kg,  $p = 0.22$ ).

A maximum portal venous pressure of 5 mmHg was used for the cold-to-warm group to increase the flows, because the vascular resistance was higher (Figure 4.4). This was kept at 3 mmHg for the HOPE+NMP group. The difference in flow in the PV did not reach statistical significance; however, values were higher for the HOPE+NMP group at the beginning of the HMP phase (280 [175–405] vs. 170 [130–300] mL/min) and at the end (320 [185–360] vs. 150

[125–305] mL/min). At the end of the NMP both groups had adequate PV flow (HOPE+NMP 1740 [1140–1675] vs. cold-to-warm 1600 [1210–1740] mL/min). Figure 4.4 portrays the vascular flow and resistance over the time of the perfusion.

**Figure 4.4: Vascular parameters throughout the perfusion protocols.**

Vascular flows and resistances along the perfusion



Panel A shows hepatic artery vascular parameters. The flow was higher for the HOPE+NMP group, despite slightly higher pressures used for the cold-to-warm group in an attempt to overcome the higher vascular resistance. A similar trend was seen for the portal vein vascular parameters (Panel B). The cold-to-warm group had higher vascular resistance mainly during the hypothermic phase, which improved during the rewarming. The higher vascular resistance required increases in the perfusion pressures, although the vascular flows were still lower during the initial period of the NMP phase. In the graphs, dots represent individual organs at the time points and the line is the median of the values for each group.



#### 4.4.6 Assessment of metabolic parameters during normothermic machine perfusion

Perfusate lactate levels were similar at the start of the perfusion (HOPE+NMP 4.7 [2.5–7.4] vs. cold-to-warm 6.6 [4.9–9.1] mmol/L,  $p = 0.31$ ). Those levels were constant for the HOPE+NMP group and increased for the cold-to-warm group, reaching higher levels at the end of the hypothermic phase (5.5 [2.4–6.9] vs. 9.8 [7.5–11.3] mmol/L,  $p = 0.01$ ). After rewarming, the lactate peak was similar between groups (HOPE+NMP 9.1 [5.7–10.0] vs. cold-to-warm 10.2 [7.9–12.3] mmol/L,  $p = 0.55$ ). Both groups metabolised lactate effectively at rewarming and NMP achieving comparable figures at the end of the perfusion (HOPE+NMP 0.8 [0.3–1.4] vs. cold-to-warm 0.7 [0.2–1.4] mmol/L,  $p = 0.84$ ).

Venous perfusate glucose was also comparable between groups at the commencement of the hypothermic phase (HOPE+NMP 10.3 [9.6–10.1] vs. cold-to-warm 11.6 [11.2–12.6] mmol/L,  $p > 0.99$ ). It increased significantly in the cold-to-warm and reached higher levels at the end of this phase (17.9 [12.7–23.7] vs. 40.0 [18.1–40.0] mmol/L,  $p = 0.07$ ). For the HOPE+NMP group, there was a slow concentration increase during the hypothermic phase and then a drop (related to the perfusate fluid replacement). After 2 hours of NMP, the livers started to consume glucose from the perfusate and both groups reached similar figures at the end of 6 hours perfusion (HOPE+NMP 27.1 [14.1–40.0] vs. cold-to-warm 24.5 [21.9–31.8] mmol/L,  $p = 0.15$ ). Perfusate pH levels were comparable between both groups throughout the entire perfusion. Bile production at 6 hours of perfusion was similar for both groups (HOPE+NMP 5.2 mL/kg liver vs. cold-to-warm 3.2 mL/kg liver). Figure 4.3 outlines these results in further detail.

Finally, all five livers submitted to the HOPE+NMP protocol were deemed viable at the end of the NMP phase and four out of five in the cold-to-warm. The non-viable liver (cold-to-warm 3)

was from a 55-year-old brain stem dead donor with a donor risk index of 2.0 and more than 13 hours of cold ischemia time. Information surrounding the fulfilment of the viability criteria are presented in Table 4.3.

**Table 4.3: Viability criteria achievement by the livers in each group.**

<b>Criteria</b>	<b>Cold-to-Warm (<i>n</i> = 5)</b>	<b>HOPE+NMP (<i>n</i> = 5)</b>
Lactate clearance ( $\leq 2.5$ mmol/L)	4 (80%)	5 (100%)
pH > 7.3 perfusate	4 (80%)	2 (40%)
Glucose metabolism	3 (60%)	4 (80%)
HA flow (> 150 mL/min)	5 (100%)	5 (100%)
PV flow (> 500 mL/min)	5 (100%)	5 (100%)
Homogeneous perfusion/ soft parenchyma	5 (100%)	5 (100%)
Bile production	3 (60%)	3 (60%)
<b>Viable liver</b>	<b>4 (80%)</b>	<b>5 (100%)</b>

**Abbreviations:** HOPE: hypothermic oxygenated perfusion of the liver; NMP: Normothermic machine perfusion of the liver; HA: hepatic artery; PV: portal vein

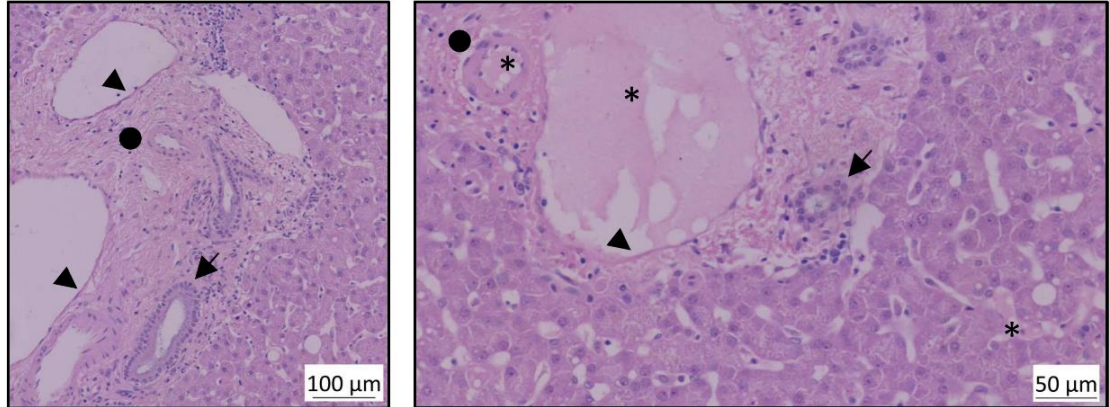
#### 4.4.7 Histological assessment

Histologically there were no signs of pre-existing acute or chronic liver disease at the beginning of the perfusion in both groups. In terms of severity of macrovesicular steatosis two livers in the HOPE+NMP were mildly steatotic (5–30%) and two were moderately steatotic (30–60%) in the cold-to-warm group. All the other organs were non-steatotic (< 5%). At the end of the 6 hours of perfusion, none of the livers exhibited areas of parenchymal necrosis, congestion or cytoplasmic vacuolization. Additional details are shown in Figure 4.5.

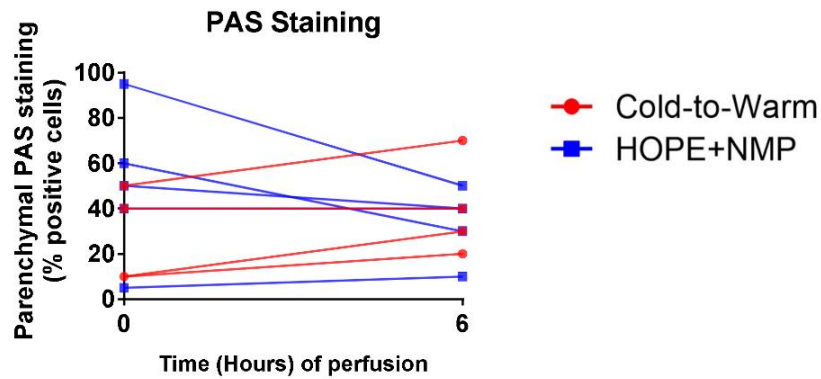
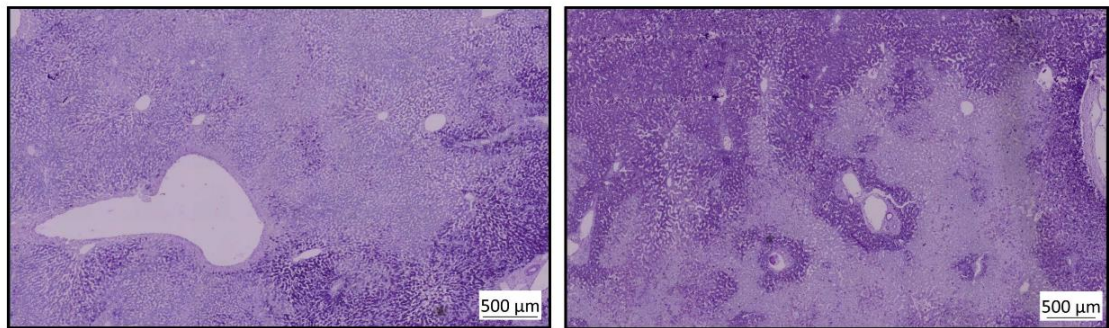
**Figure 4.5: Histological assessment of the perfused organs.**

## Histological assessment

**A**



**B**



Panel A shows two haematoxylin–eosin (H&E)-stained paraffin sections at the end of the perfusion run. The left-hand picture is a large portal tract showing well preserved bile duct (arrow), artery (circle) and portal vein (arrowhead). A similar picture was seen for smaller intrahepatic portal tracts in the right-hand picture, where the same well-preserved structures can be identified; and, interestingly, the presence of the HBOC-based perfusate can be visualized (asterisk) in the vein, artery and even in the sinusoids. Panel B shows the change in glycogen content over time. The first figure on the left shows one liver from the cold-to-warm group at time 0 with severely depleted glycogen stores; at the end of 6 hours of perfusion, this was slightly replenished. The graph compares the dynamic changes in glycogen content between groups.

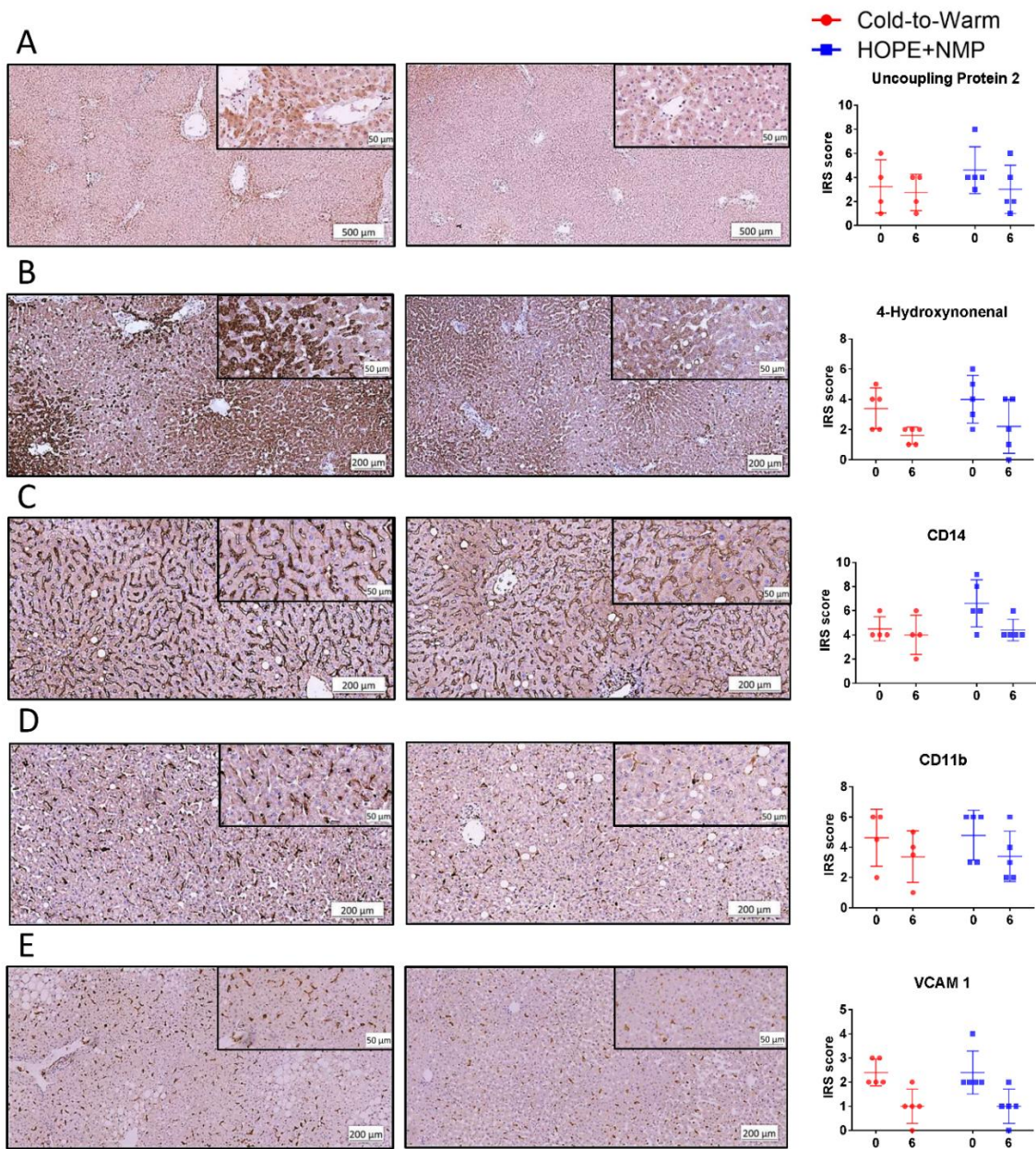
#### 4.4.8 Immunohistochemical assessment of oxidative stress and tissue inflammation

The expression of tissue markers of oxidative injury and activation of inflammatory cells were comparable for the two groups at the start of the perfusion. During the 6 hours of perfusion, there was an overall decrease in the tissue expression of UCP2, a marker associated with mitochondrial ROS production, (HOPE+NMP  $\Delta$ IRS: -2.0 [IQR: -2.5/ -0.5] vs. cold-to-warm  $\Delta$ IRS: -1.0 [-2.7/ 2.2],  $p = 0.48$ ) and 4-HNE, a product of the lipid peroxidation of cells (HOPE+NMP  $\Delta$ IRS: -2.0 [-2.0/ -1.5] vs. cold-to-warm  $\Delta$ IRS: -1.5 [-2.0/ -1.0],  $p = 0.43$ ). These markers were present mainly in hepatocytes. Considering downstream activation of the inflammatory cascade, the expression of CD14, part of the signalosome of the toll-like receptor 4, decreased in the HOPE+NMP group and was stable for the cold-to-warm, however, the difference was not statistically significant ( $\Delta$ IRS: -2.0 [-3.5/ -1.0] vs.  $\Delta$ IRS: 0 [-1.5/ 0],  $p = 0.08$ ). Tissue inflammatory response was assessed by the expression of CD11b on activated polymorphonuclear leukocytes (neutrophils and monocytes/macrophages) and VCAM-1. Its levels of expression decreased similarly between the two groups for both CD11b (HOPE+NMP  $\Delta$ IRS: -1.0 [-2.5/ -0.5] vs. cold-to-warm  $\Delta$ IRS: -1.0 [-1.7/ -1.0],  $p = 0.80$ ) and VCAM-1 (HOPE+NMP  $\Delta$ IRS: -1.0 [-2.0/ -1.0] vs. cold-to-warm  $\Delta$ IRS: -1.5 [-2.0/ -1.0],  $p = 0.85$ ). Data are presented in Figure 4.6.



**Figure 4.6: Immunohistochemical assessment of oxidative tissue injury and activation of the inflammatory cascade.**

Immunohistochemical assessment of markers of oxidative injury and inflammation



Panel A shows the tissue expression of the uncoupling protein 2, a marker for reactive oxygen species (ROS) generation; the left-hand picture shows a moderate diffuse reaction of the staining at time 0, mainly localised at peri-portal areas, which changed to a mild reaction at the end of the 6 hours perfusion. The graph on the right shows the variation in the two groups at these time points. This downregulation in ROS production is associated with a reduction in tissue expression of 4-hydroxynonenal (4-HNE) (Panel B), an established marker for lipid peroxidation during oxidative stress. The lowered oxidative injury decreases the tissue expression of the cluster of differentiation (CD)14 in macrophages (Panel C).

CD14 is part of the signalosome of the toll-like receptor 4, which in turn leads to the activation of inflammatory and endothelial cells, perpetuating and aggravating tissue injury. In accordance, a downward trend can be seen during the perfusion in the presence of activated leukocytes, identified by the expression of the CD11b (Panel D) and activated endothelial cells, as represented by the expression of the vascular cell adhesion molecule 1 (VCAM-1) (Panel E). Therefore, both combined protocols exhibited a similar decreasing trend in the expression of markers of ischaemia-reperfusion injury during the perfusion. In the graphs, dots represent individual organs at the time points, and the line is the median of the values for each group.

## 4.5 Discussion

In the present study we have shown the feasibility of using an HBOC-based perfusate for a combined protocol of cold to warm *ex situ* MP. Whilst the use of a single perfusate throughout the entire perfusion simplified the logistics of combining HMP and NMP with a slow and controlled rewarming period, it was associated with high vascular resistance and low flow dynamics. However, this difference did not affect either tissue oxygenation or the enhancement of the bioenergetic status of the organs during the hypothermic phase and allowed effective recovery of the metabolic function of the organs during the normothermic phase.

Our group has recently shown that the combination of a short-term period of HOPE can improve the metabolic parameters of discarded high-risk ECD livers, as evaluated by a subsequent period of NMP (11). Whilst HMP replenishes cellular energy stores and mitigates ischaemia-reperfusion injury, NMP offers full metabolic support to donor organs allowing metabolic function assessment. Importantly, tests to assess liver function during HMP are still not readily available. The use of nuclear magnetic resonance spectroscopy to determine the bioenergetic status of organs during perfusion or to analyse perfusate composition are possibilities to be further investigated (9, 31, 32). The inability to utilise a red blood cells-based perfusate during HMP as well as the need for an oxygen carrier during NMP impose the need for a perfusate exchange during the perfusion process if one wishes to combine both techniques. HBOC has

the advantage that it can potentially be used under a wide range of temperatures with reports on subnormothermic MP at 20 °C and NMP (14, 16).

A previous study that performed D-HOPE on human livers using UW-MP as perfusate reported, at 2 hours of perfusion, median flows of 365 mL/min for PV and 84 mL/min for HA (8). Our experiments utilised the same perfusion device and applied the same protocol in terms of perfusion pressures and temperature for the D-HOPE phase. However, using an HBOC-based perfusate we accomplished lower median vascular flows at 2 hours of D-HOPE (150 mL/min and 42 mL/min, respectively). Median vascular arterial resistance at the same time point was proportionally higher for the livers of our experiment (0.4 mmHg/mL/min/kg) than with this previous study (approximately 0.2 mmHg/mL/min/kg). These findings suggest a possible association between the use of an HBOC-based perfusate at 10 °C, higher vascular resistance and reduced flow.

The association between HBOC and impaired microcirculation has been reported previously and diverse theories were proposed in an attempt to explain this (33). It has been suggested that, based on their small size, HBOCs molecules may be able to permeate into the subendothelial space and acellular haemoglobin consumes the nitric oxide (NO) produced by endothelial cells, thereby preventing its biological vasodilatory effect (34, 35). Alternatively, the low viscosity of HBOCs may affect the regulation of flow within the microcirculation (36, 37). Mechanistically, the lower shear stress may potentially decrease the production of NO and, therefore, have an impact of vasoconstriction (33, 38). Finally, the autoregulatory theory proposes that terminal arterioles adjust flow according to needs based on PO<sub>2</sub> levels (39, 40). HBOC would potentially be delivering high concentrations of oxygen at this site paradoxically triggering vasoconstriction. Whether these theories explain the effect observed in our study remains to be seen and requires further investigation. The pO<sub>2</sub> used for the referred UW-MP D-

HOPE study (60 kPa) (8) was the same as that for our HBOC-based perfusate. However, we do not have comparative data regarding the amount of oxygen delivered at the level of terminal arterioles in each of these models. In addition, in this study we are unable to comment on the difference in viscosity between Hemopure<sup>®</sup> and UW-MP at 10 °C. Thus far, the theory that acellular haemoglobin scavenges the NO produced in the subendothelial space remains plausible; however, MetHb levels were stable during HMP, contrary to what would be expected – NO binds to the oxygen sites in haemoglobin, thus generating MetHb.

More importantly, despite these differences in vascular parameters, the enhanced oxygen delivery of the HBOC is likely to have balanced it because adequate oxygen supply was offered to the cells. Both groups showed a reduction in mitochondrial respiration, as represented by a fall in the oxygen requirements associated with lowered CO<sub>2</sub> production during the hypothermic phase. These findings were associated with enhanced mitochondrial oxidative function and ATP synthesis. This improved mitochondrial oxidative function resulted in lower oxidative tissue injury and activation of inflammatory cells mitigating reperfusion injury during rewarming. Similar findings were reported previously using HOPE and D-HOPE techniques without the use of an oxygen carrier in the perfusate (8, 9). The initial lactate levels differed slightly between groups at the beginning of the perfusion as a result of different perfusate composition. Whilst those levels were flat during HOPE, they increased slightly during the first hour of D-HOPE. A similar initial trend was described in previous publications (7, 8). Importantly, this initial elevation is most likely related to the initial flush out of the organs because in isolation, without other markers of reduced perfusion, lactate does not reflect anaerobic metabolism.

Previous clinical studies have reported minor increases in MetHb levels after transfusion of Hemopure<sup>®</sup> in humans (41, 42). MetHb is an oxidized form of haemoglobin that cannot bind



oxygen and has a reduced ability to release oxygen to tissues (43). Physiologically, erythrocytes' enzymes reduce the MetHb formed, keeping levels lower than 2% (44). Clinically, levels under 15% are only associated with a greyish skin without further complications (43, 44). In the context of MP, a previous report on a porcine model showed stable MetHb levels around 2% during subnormothermic MP (16). We established similarly stable levels during HMP and rewarming, nevertheless, during NMP, MetHb levels increased steadily at a similar rate for both groups, reaching median levels of around 7% at the end of the perfusion. Despite this increase, there was no suggestion of any ischaemic injury to the livers until 6 hours of perfusion.

To explore fully the advantages of having a perfusate that could be used in a wide range of temperatures, we incorporate a COR period in the HBOC-based perfusate group. This technique permits avoiding subtle changes in the temperature of the organs and was shown to be beneficial for mitochondrial function yielding optimized results in a clinical study (17). We have employed the same protocol as that clinical study in our experiments, with the difference of the HBOC-based perfusate instead of the Custodiol-N (Dr. Köhler Chemie, Bensheim, Germany) used by the original authors. During this phase, no significant differences were seen in terms of perfusion parameters or liver metabolism features.

The current work has also shown that while an oxygen carrier-free-based perfusate was equally effective during the hypothermic phase, it is feasible to use a single HBOC-based perfusate within a range of temperatures in the setting of MP of donor livers. This can be logistically advantageous considering the combination of HMP and NMP techniques, because it eliminates the need for perfusate exchange and can increase the clinical applicability of the combined protocol. Despite an effect on flow dynamics with higher vascular resistances and lower flows, the enhanced capacity of oxygen delivery of Hemopure<sup>®</sup> potentially prevented any harmful

effects to the organs. Such findings facilitate and support the safety of the use of a combined protocol of MP that can improve the metabolic functions of high-risk discarded ECD livers. The Groningen group recently reported the transplantation of one donor liver after performing a similar protocol of D-HOPE+COR+NMP using a single HBOC-based perfusate (45). The authors perfused 3 donor livers discarded for transplantation performing D-HOPE followed by COR and then assessed organ function within 150 minutes of NMP. Only one organ was deemed viable and transplanted with the recipient doing well after 3 months of transplantation. The authors conclude that combination of these perfusions using a single HBOC-based perfusate is feasible (45).

The present study does have limitations. First, the organs were not transplanted, therefore viability remains theoretical, and the singularity of human donor livers makes the experimental groups imperfectly matched. The uninterrupted cold-to-warm MP protocol using an HBOC-based perfusate was compared with a previously published combination of HMP+NMP, and the differences in the perfusate composition and technique employed limited isolated comparison of HMP techniques exclusively. We were unable to evaluate transaminases in the perfusate, traditionally used as markers of hepatocellular injury, because our clinical laboratories could not perform the analysis and we could not retrieve reliable concentrations from rethawed frozen samples. Moreover, the absolute values and proportional ATP increase were lower than those reported by other studies performing D-HOPE on human livers (8). This is most likely related to a longer cold ischaemia time in our study, because this can underestimate ATP readings.

#### **4.6 Conclusion**

This study shows that despite changes in flow dynamics and vascular resistance, the use of an HBOC-based perfusate in a wide range of temperatures during *ex situ* MP of the liver is feasible and not deleterious to the organs. The use of a single perfusate facilitated the combination of HMP and NMP in an uninterrupted fashion, from cold to warm. It eliminates entirely any concerns of exposing the organs to additional unnecessary ischaemia time required for perfusate exchange and thus increases the clinical applicability of the combined protocol of hypothermic and normothermic perfusions. This approach, in turn, can finally enhance the rescue of metabolic parameters of high-risk ECD livers to acceptable standards for transplantation.

## **4.7 Appendices**

### **4.7.1 Supplementary Methods**

#### **4.7.1.1 Immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded sections using Specie specific ImmPRESS™ (Peroxidase) Excel Amplified HRP Polymer Staining Kit. The primary antibodies used were:

- anti-uncoupling protein 2 (UCP2) antibody (ab203244 – Abcam, Cambridge, UK) rabbit polyclonal at 1/600 dilution.
- anti-cluster of differentiation 14 (CD14) antibody (ab36595 – Abcam, Cambridge, UK) mouse monoclonal at 1/500 dilution.
- anti-cluster of differentiation 11b (CD11b) antibody (ab52478 – Abcam, Cambridge, UK) rabbit monoclonal at 1/200 dilution.
- anti-4-hydroxynonenal (4-HNE) antibody (ab46545; Abcam, Cambridge, MA, USA) rabbit polyclonal IgG at 1:200 dilution.
- anti-vascular cell adhesion molecule 1 (VCAM-1) antibody (SAB1406579 - Sigma-Aldrich Inc, St Louis, MO, USA) mouse polyclonal at 1/400 dilution.

All sections were wholly digitalized using the Axio Scan.Z1. Four areas at a magnification of 400x were randomly selected using the ZEN image analysis software. Areas of tissue edge were not included due to artefacts that could confound the analysis. Thereafter, the four random images were analysed for overall expression of the staining.

#### **4.7.1.2 Adenosine trisphosphate quantification**

Adenosine trisphosphate (ATP) concentration was determined using the ATP Bioluminescent Kit (FL-AA - Sigma-Aldrich Inc, St Louis, MO, USA). One-hundred milligrams of frozen

liver tissue was immediately homogenised in 1 mL SONOP Buffer (0.372g EDTA in 130 mL ddH<sub>2</sub>O [adjusted to pH 10.9 with NaOH] = 370 mL of 96% Ethanol) using the GentleMacs system. Particulates were removed by centrifugation at 13,000xg. The protein concentration was determined in the supernatant with the use of a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, Illinois, USA) and the concentration adjusted to 300 µg/mL protein with the SONOP buffer. Samples were then diluted 10-fold in 100 µM phosphate buffer and then the ATP concentration determined. Concentrations were determined from a calibration curve on the same plate, corrected for amount of protein and expressed as nmol/g protein.

#### **4.7.1.3 Oxygen content in the perfusate equation**

$O_2 \text{ content} = (pO_2 \times K) + (sO_2 \times Hb \times c)$ ,  $pO_2$  in kPa,  $K$  equals 0.027 for  $O_2$  in water at 37 °C,  $Hb$  in mmol/L and  $c$  equals 91.12 mL $O_2$ /mmol for the oxygen binding capacity of haemoglobin.

#### 4.7.1.4 Supplementary Table S4.1

**Table S4.1: Machine perfusion fluid constitution**

1000 mL (4 units) acellular oxygen haemoglobin carrier Hemopure® (haemoglobin glutamer-250-bovine; HBOC-201, Hemoglobin Oxygen Therapeutics LLC, Cambridge, MA)
1000 mL 5% w/v human albumin solution (Alburex 5, CSL Behring GmbH, Germany)
10000 IU heparin (Wockhardt, UK)
30 mL sodium bicarbonate 8.4% (B. Braun Medical Limited, UK)
10 mL calcium gluconate 10%
500 mg vancomycin (Wockhardt, UK)
60 mg gentamicin (Cidomycin, Sanofi, UK)
50 mL 10% v/v Aminoplasma (B. Braun Medical Limited, UK)
0.2 mL Cernevit (Baxter Healthcare Ltd., UK)
0.1 mg phytomenadione (Konakion, Roche Products Ltd, UK)
Epoprostenol (Flolan, GlaxoSmithKline, UK, 2 µg/mL) continuous infusion commencing at 4 mL/hour

**Abbreviations:** MA- Massachusetts; UK- United Kingdom.

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## Chapter 5

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# ***EX SITU* MACHINE PERFUSION AS A TOOL TO RECONDITION STEATOTIC DONOR LIVERS: TROUBLESOME FEATURES OF FATTY LIVERS AND THE ROLE OF DEFATTING THERAPIES. A SYSTEMATIC REVIEW**

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### **Published article**

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Y.L.B. designed the study, performed the literature review, interpreted the data and wrote the manuscript. A.P.C.S.B. assisted with the literature review and analysis. J.A., H.M., D.F.M. and R.H.B. reviewed critically the manuscript and assisted in editing it. S.C.A. reviewed critically the manuscript, assisted in the interpretation of the data and editing the manuscript.

## 5.1 Abstract

Longstanding research has shown that increased lipid content in donor livers is associated with inferior graft outcomes post-transplantation. The global epidemic that is obesity has increased the prevalence of steatosis in organ donors, to the extent that it has become one of the main reasons for declining livers for transplantation. Consequently, it is one of the major culprits behind the discrepancy between the number of donor livers offered for transplantation and those that go on to be transplanted. Steatotic livers are characterised by poor microcirculation, depleted energy stores due to an impaired capacity for mitochondrial recovery and a propensity for an exaggerated inflammatory response following reperfusion injury culminating in poorer graft function postoperatively. *Ex situ* machine perfusion, currently a novel method in graft preservation is showing great promise in providing a tool for the recovery and reconditioning of marginal livers. Hence, reconditioning these steatotic livers using machine perfusion has the potential to increase the number of liver transplants performed. In this review, we consider the problematic issues associated with fatty livers in the realm of transplantation and discuss pharmacological and non-pharmacological options that are being developed to enhance recovery of these organs using machine perfusion and defatting strategies.

## 5.2 Introduction

Liver transplantation (LT) is currently the only curative option for end-stage liver disease, or cirrhosis, and early-stage hepatocellular carcinoma (1, 2). However, there is a continuing discrepancy between organ availability and quality and the ever-increasing number of patients eligible for LT (3–6). One potential method to bridge this discrepancy is the increased utilisation of extended-criteria donors (ECD) or marginal donors (3). These donor livers include fatty livers/steatotic livers (referred to as steatotic livers hereafter), donors with advanced age, donor livers with prolonged cold ischaemia times (CIT) and donation after circulatory death (DCD). Transplantation of ECD livers is associated with a higher incidence of graft dysfunction, including primary graft non-function (PNF), and inferior patient survival after LT (5, 7, 8). Recently *ex situ* machine perfusion (MP) of ECD livers has generated considerable interest with early preclinical and clinical series suggesting it may improve organ preservation, as well as allowing real-time organ function assessment (9, 10). Indeed, many in the transplant community are suggesting that MP may be a potential method to increase the donor pool (11). Importantly, steatosis is the primary reason for declining donor livers in the United Kingdom as it is in other countries (12, 13). Hence the potential use of MP to reduce steatosis in donor livers and/or improve its metabolic function may be a useful avenue to improve organ utilisation for transplantation.

Despite clinical need the use of MP for steatotic livers still relatively under-researched. The objective of this review was to investigate how MP could be a tool to expand the use of steatotic organs in LT focusing specially on steatosis reversal (more commonly known as defatting).

## 5.3 Methods

The following databases were searched for the development of this review: PubMed, Web of Science, EMBASE, MEDLINE, Cochrane Library and Scopus. The literature search was performed until March 1, 2018 and the medical subject heading (MeSH) terms and Emtree keywords ‘machine perfusion, liver transplantation’ combined with the free text terms ‘steatosis’, ‘fatty livers’, ‘defatting’ and ‘steatosis reversal’. The search strategy used is shown in supplementary Table S5.1. There was no initial limit of date for inclusion in an attempt to identify most of the papers published.

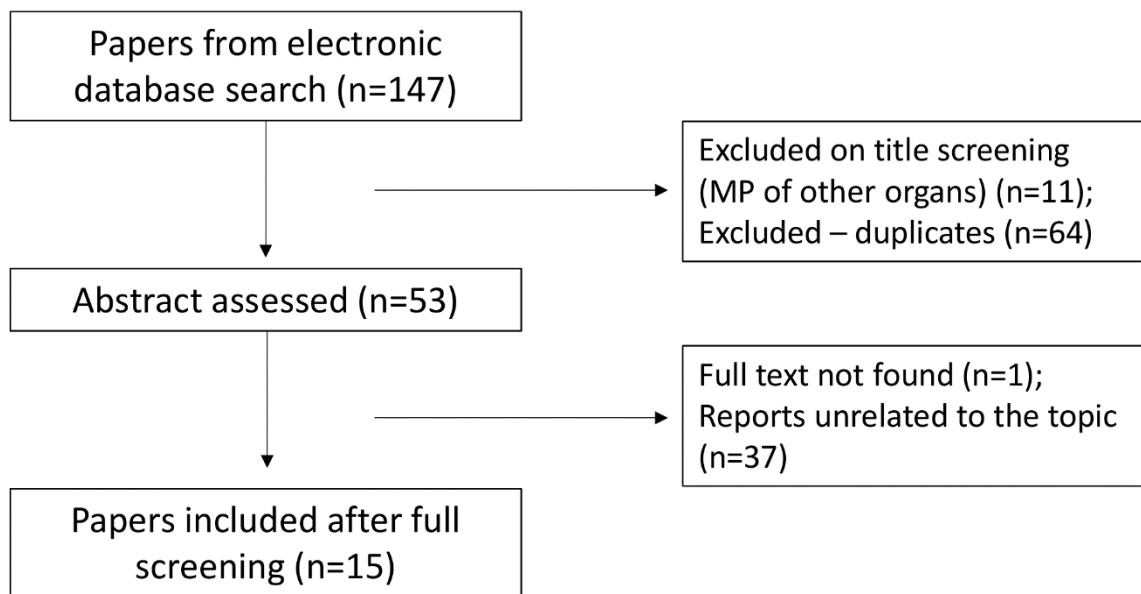
Two authors (Y.L.B. and A.P.C.S.B) selected the articles independently to include in the review according to the Preferred Reporting Systematic Reviews and Meta-Analyses (PRISMA) protocol (14). Initially article titles that completely did not match the aims of this review were excluded and the remaining abstracts analysed. In this second step abstracts identified as not relevant were excluded. Finally, full papers that could be retrieved were analysed and included in the review. Figure 5.1 provides a flow diagram of the selection process.

Inclusion criteria were: (1) articles studying MP and steatotic livers; (2) animal models or human liver experimentation; (3) *In vitro* models of intracellular fat loading and/or defatting; and (4) articles written in English and published. Exclusion criteria were: (1) studies with MP not involving steatotic livers; (2) studies on MP of other organs; and (3) articles not written in English.

The reported impacts of MP on steatotic livers were critically analysed. Studies were assessed by the authors in terms of study design, methods and outcomes. A focus was given on defatting strategies due to their promising potential significance. No assumptions or simplifications were made, and risks of biases identified for individual studies are discussed in this review. Background involving steatosis and the mechanisms of action of the drugs were further

investigated in a non-systematic way as it was not the primary objective of the review, however the authors felt it was essential for readers to fully understand the field. No review protocol was registered before starting this manuscript.

**Figure 5.1: Study flow diagram for systematic review of the literature on *ex situ* machine perfusion of the liver and steatosis/ defatting.**



After initial literature search duplicates articles were removed and titles screened, if related to other machine perfusion (MP) of other organs they were excluded. Remaining abstracts were then assessed and excluded if not related to the topic. Full papers found were then included in the study.

## 5.4 Results

Fifteen articles met our inclusion/ exclusion criteria and are described hereafter. The complete screening process is presented in Figure 5.1.

### 5.4.1 Steatosis

Obesity is a global issue and according to the World Health Organization it is responsible for 2.8 million deaths per year. In 2008, the global prevalence of obesity (i.e. BMI  $\geq 30$  kg/m<sup>2</sup>) was estimated to be 10% for men and 14% for women (15).

Obesity is linked to the metabolic syndrome and its hepatic manifestation is steatosis known as Non-Alcoholic Fatty Liver Disease (16). At the cellular level, steatosis is characterised by the accumulation of triacylglycerol in the cytoplasm of hepatocytes. Insulin resistance plays a central role leading to increased lipolysis in adipocytes and therefore an increased availability of free-fatty acids (FAs) to the liver. Furthermore, obesity is associated with increased release of inflammatory cytokines (e.g. IL-6 and TNF- $\alpha$ ) and low levels of adiponectin (17).

#### **5.4.1.1 Steatosis Classification and Grading**

Histologically, steatosis is classified as follows: (1) ‘macrovesicular steatosis’ (MaS), which describes the presence of a large lipid droplet (LD) within the hepatocyte cytoplasm which displaces the nucleus; and (2) ‘microvesicular steatosis’ (MiS), when numerous small LDs can be found in the cytoplasm without any alteration in the position of the cell’s nucleus. In ‘pure’ MiS small LDs uniformly occupy the whole cell. The former is a rare condition associated with specific conditions (e.g. Reye’s syndrome, acute fatty liver of pregnancy and mitochondrial hepatopathies) and such livers would not be considered for donation (18). However, the presence of areas of MiS have been described in non-alcoholic and alcoholic liver disease (19). Mixed forms of large and small LDs are common in steatotic donor livers and classified as MaS (18, 20). Later the terms large-droplet and small-droplet MaS were created to differentiate mixed forms (21). Large-droplet MaS was used for single LD larger than half of the cell displacing the nucleus; and, small-droplet MaS if smaller than half of the cell and not displacing the nucleus (21). Whilst these new subcategories may better classify steatosis, their nature in

relation to pathology remains unclear with most studies reporting just the presence of MaS and MiS.

Traditionally MaS has been graded according to the percentage of cells infiltrated by fat as mild  $\leq 30\%$ , moderate 30–60% and severe  $\geq 60\%$ . However, there is still no consensus in the literature on the standardisation of methods for sample collection or analysis to quantify hepatic steatosis what may cause discrepancy in results between different studies (22).

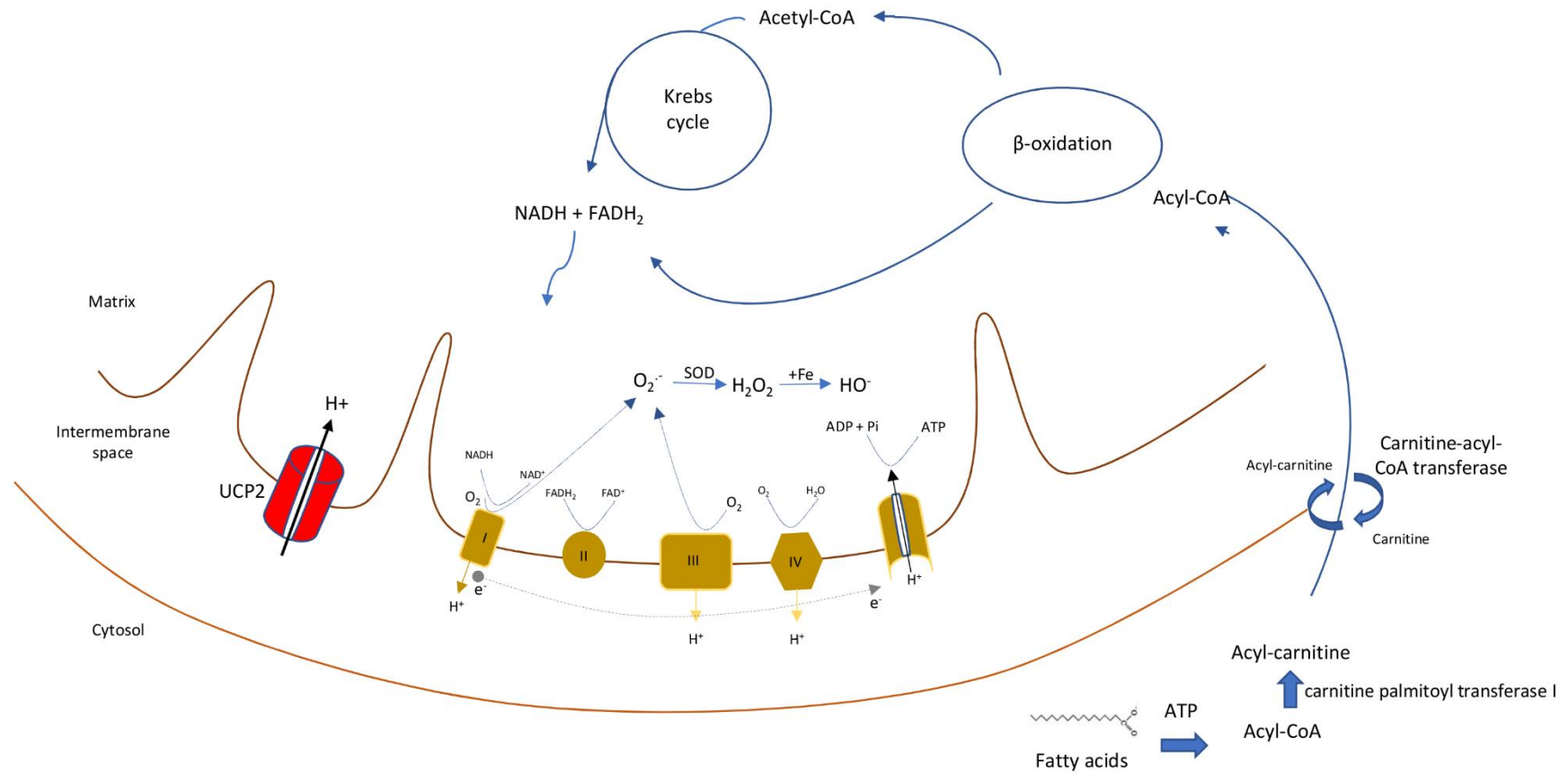
#### **5.4.1.2 The cellular effects of steatosis**

Steatosis has several effects upon cellular metabolism. Adenosine triphosphate (ATP) synthesis is impaired and it has been linked to impaired mitochondrial ATP synthase activity (23). When exposed to stressful stimuli such as ischaemia-reperfusion injury (IRI) these livers demonstrate increased injury relative to non-steatotic livers (24–26). Diminished ATP stores have also been correlated with higher rates of necrosis in steatotic livers during IRI (27).

The mitochondrion plays an integral part in regulating the fate of steatotic hepatocytes. In a normal lean liver, the inner-membrane mitochondrial protein Uncoupling Protein 2 (UCP2) acts as a mitochondrial antioxidant, dissociating oxidative phosphorylation from ATP production (28). UCP2 is overexpressed in the mitochondria of fatty livers, promoting increased proton leakage (29) and it has a direct relationship with Reactive Oxygen Species (ROS) production (27). Furthermore, UCP2 was also related to necrotic cell death, stalling the cell cycle at the G1 phase and interrupting apoptotic pathways as well as hepatic regeneration (28). Figure 5.2 illustrates the complex relationship between UCP2 and the mitochondrial electron transport chain.



**Figure 5.2: The  $\beta$ -oxidation of fatty acids in the mitochondria.**



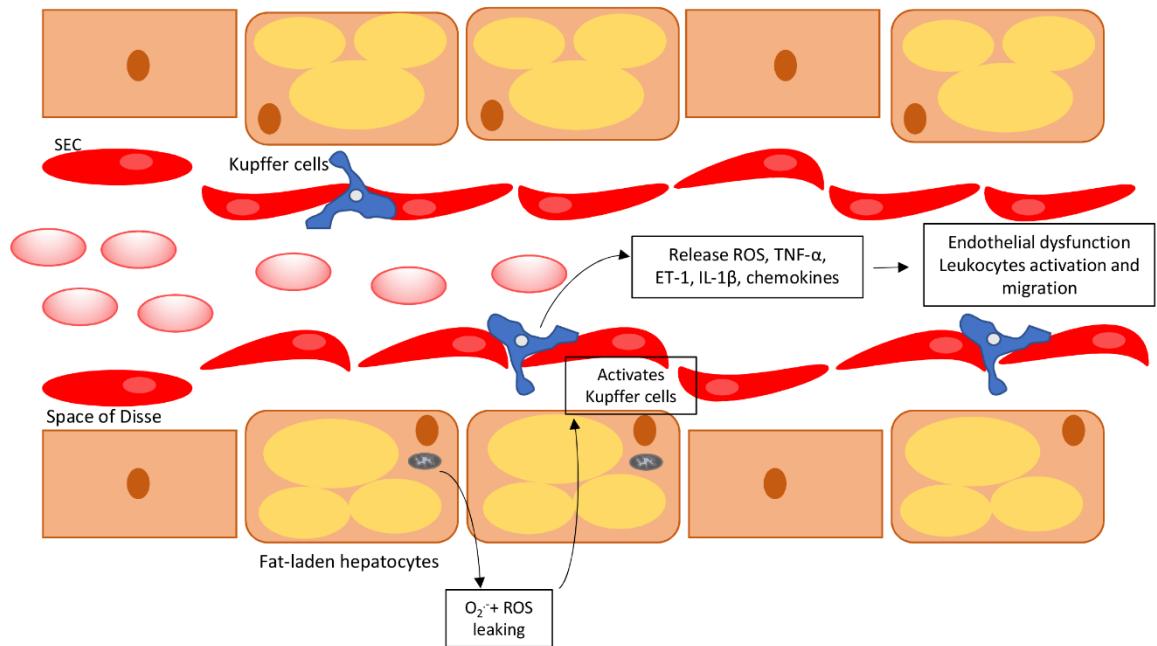
**Figure 5.2: The  $\beta$ -oxidation of fatty acids in the mitochondria.** Cytosolic fatty acid reacts with ATP producing fatty acyl adenylate which in turn react with free coenzyme A originating fatty acyl-CoA. Carnitine palmitoyltransferase I catalyse the reaction of acyl-CoA with the hydroxyl group of carnitine by carnitine palmitoyltransferase. Acyl-carnitine is transported inside by a Carnitine-acyl-CoA transferase and a carnitine is transferred outside. Acyl-CoA is processed by  $\beta$ -oxidation releasing acyl-CoA molecule 2 carbons shorter than it was at the beginning of the process, acetyl-CoA, NADH and FADH<sub>2</sub>. Acetyl-CoA will undergo to the cycle of Krebs releasing more NADH and FADH<sub>2</sub>. Electrons from NADH and FADH<sub>2</sub> will be utilised in the electron transport chain transferring electrons from donors to recipients, and this movement of electrons is linked with transfer of protons (H<sup>+</sup>). This electrochemical proton gradient generated is essential in the production of adenosine triphosphate (ATP). Complex I (NADH dehydrogenase) and complex III (coenzyme Q) generates superoxide (O<sup>2.-</sup>), which interacts with superoxide dismutase producing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). H<sub>2</sub>O<sub>2</sub> in the presence of transition metals generates hydroxyl radical (HO<sup>-</sup>). Hydroxyl radical causes lipid peroxidation in membrane phospholipids, DNA injury that could culminate with cell death. Lipid peroxidation leads to the production of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) which are eliminate from damaged hepatocytes into the space of Disse. UCP2 is overexpressed in the mitochondria of fatty livers promoting increased leakage of protons (H<sup>+</sup>) which dissipates the mitochondrial membrane potential and dissociates the electron transport chain from the oxidative phosphorylation. Lowering the proton gradient UCP2 decreases the generation of superoxide anion at the complex 1 of the electron transport chain.

#### 5.4.1.3 The microcirculation of steatotic livers

Early studies using steatotic rodent livers retrieved and stored cold for 6 hours demonstrated damage to the hepatic microcirculation characterised by narrowing of the hepatic sinusoids relative to lean controls (30). These changes are due to a reduced sinusoidal diameter secondary to compression by fat-laden hepatocytes (26, 31–34) and/or obstruction of the microcirculation by cells or debris as verified by Doppler studies (33–37). Indeed, the degree of steatosis correlates with reduced total blood flow as well as the hepatic parenchymal microcirculation in livers histologically classified as moderately or severely steatotic (37).

Microcirculatory dysfunction leads to a ‘secondary’ ischaemic hit during reperfusion, allowing the production of more ROS and activation of Kupffer cells (38). Activated Kupffer cells produce endothelin-1, a vasoconstrictor, in excess of the counterbalance provided by nitric oxide (NO), a vasodilator. Lastly, cytokines released cause endothelial dysfunction with expression of adhesion molecules (E-selectin, VCAM-1 and ICAM-1) and activation, migration and accumulation of platelets and leukocytes (39, 40). A summary of these mechanisms is provided in Figure 5.3.

**Figure 5.3: Mechanisms of interaction between fatty loaded hepatocytes and the other cell types of the liver.**



Microcirculatory dysfunction in fatty livers during reperfusion is a multi-factorial process involving complex metabolic pathways. Firstly, ballooned lipid-laden hepatocytes cause reduction in sinusoidal diameter that causes a mechanical effect worsening the flow in steatotic livers. During the restoration of blood flow, after periods of ischaemia, oxygen offer overcomes the capacity of the mitochondrial electron transport chain to recover. Consequently, there is a leakage of electrons that reacts with oxygen and generate reactive oxygen species (ROS) (superoxide anion, hydrogen peroxide). Kupffer cells get activated by this production and start a sustained production of endothelin-1 (ET-1) a potent vasoconstrictor associated with inflammatory mediators including ROS, tumour necrosis factor- $\alpha$  ( $TNF-\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and chemokines. All these mediators damage the sinusoidal endothelial cells (SEC) and activate the expression of adhesions molecules which in turn cause accumulation of platelets and leukocytes. Hence, local and systemic inflammatory response develop with hepatocellular damage.

#### **5.4.1.4 The pro-inflammatory microenvironment within steatotic livers**

In experiments using a mouse model of endotoxin liver injury, Kupffer cells from steatotic livers were shown to exhibit lower phagocytic activity and overproduction of ROS, interleukin-6 and interleukin-1 $\beta$ , when compared to lean controls (41). These observations were associated with increased endothelial dysfunction and activation of neutrophils. Persistent activation of these cells enhances the production of ROS and proteases which aggravate the hepatocellular injury (23, 42).

#### **5.4.1.5 From bench to bedside: Rapid deterioration of steatotic livers during ischaemia and the impact on liver transplantation**

The cellular changes of steatotic livers described above are probably the main reason for their increased vulnerability to static cold storage (SCS). Briefly, when exposed to ischaemia during SCS the cells consume the diminished ATP stores and then struggle to replenish it due to impaired mitochondrial functioning associated with uncoupling of the electron transport chain, as described above. The resulting generation of ROS causes oxidative injury and mediates the activation of the inflammatory response leading to tissue damage. Those problems are exacerbated by impaired microcirculation perpetuating ischaemia even after reperfusion and worsening the cellular injury induced by the reperfusion resulting in cell death. Altogether those factors contribute to an exacerbated IRI and an impaired early functional recovery.

#### **5.4.1.6. Impact of Steatosis on Liver Transplantation**

MiS has been shown to be less deleterious in the setting of liver transplantation. Livers with even more than 60% of MiS were shown to be safe for transplantation in terms of patient and graft survival and a feasible option to expand the pool of donors (43, 44). This finding can in

part be explained by less mechanical compression of the sinusoids by hepatocytes laden with smaller LDs (45).

Early experience with steatotic livers in transplantation was poor with clinical series demonstrating high incidence of PNF (13, 30, 46–50). Mildly steatotic livers are widely considered to be safe for transplantation as there is no evidence to support increased risk of postoperative complications with the use of these grafts (51). Studies on the use of moderately steatotic livers describe varying outcomes, particularly with respect to graft survival and early graft dysfunction (52–59). Westerkamp *et al.* 2015 reported results of transplantation of 19 moderately steatotic livers compared with non-steatotic in the context of short CIT (< 8 hours) (60). Similar patient and graft survival were reported with an increase in the rate of postoperative complications and markers of hepatic injury (60). A summary of these studies is presented in Table 5.1, the lack of standardisation for the timing of liver biopsy, type of biopsy (core needle or wedge), staining performed, and definition of MaS is evident. It is important to note that these studies employed strict selection criteria for moderately steatotic donor livers keeping other associated risk factors to a minimum, such as the exclusion or avoidance of moderately steatotic DCD livers, elderly donors, selection of recipients that may cope better with possible delayed graft functioning and consideration of only steatotic organs with short CIT (6–8 hours) (61). Salizzoni *et al.* 2003 followed a cohort of 64 donors after brain death (DBD) with more than 15% of MaS and concluded that CIT greater than 10 hours, donor age > 65 years old and a hepatitis C positive status in the recipient were predictive factors for poor prognosis after transplantation (62). Applying these criteria to the UK donor pool would significantly limit the expansion of the donor pool, thereby justifying investigation of new strategies to increase the usability of steatotic organs.

Severely steatotic livers are usually declined for transplantation because of the risk of PNF and poor prognosis. However, McCormack *et al.* 2007 followed the outcomes of 20 severely steatotic DBD transplanted livers (> 90% total steatosis) and showed excellent results, although only 2 grafts had more than 60% MaS (63). Wong *et al.* 2016 analysed 19 biopsies of severely steatotic livers (defined as > 60% large fat droplets occupying more than half of the hepatocyte, usually causing displacement of the nucleus of the cell) from DBD donors and showed no early allograft dysfunction post-transplant and a 3-year survival rate of 94.7% (64). They go on to emphasise, however, that these results were based on low risk donors and short CIT (64). Although these authors have suggested that transplantation of those livers can yield acceptable results, these series should be treated with caution because of variability in steatosis quantification and the constraints stated by the authors.

In summary, the current literature suggests that MaS does have a negative impact on outcomes following transplantation although the studies have variation in the methodology used (42, 44, 61, 65). To date, the use of these organs relies heavily on the absence of other criteria of donor marginality. However, for clinicians an aging population associated with increasing rates of obesity requires the development of strategies to maximise the utilisation of these grafts from the donor pool.

**Table 5.1: Characteristics of clinical studies reporting transplantation of moderately macrovesicular steatotic livers.**

First author	Year	Number of cases	MaS (%)	Steatosis assessment method/ definition	Donor type	Median CIT (minutes)	Outcomes
Wong <i>et al.</i> (64)	2016	19	60%	Post-reperfusion core needle biopsy. H&E staining. MaS: large lipid droplets (more than half of the cell) displacing the nucleus.	DBD	384	3-year survival 94.7%; 0% of early allograft dysfunction
Westerkamp <i>et al.</i> (60)	2015	19	30–60%	Core needle biopsy performed during the bench. H&E staining. MaS: fatty vesicles larger than and often displacing the nucleus of the cell.	DBD and DCD	437	1-year patient survival of 95%; 1-year graft survival 84%; 1 case of primary non-function; Higher incidence of postoperative severe complications
Doyle <i>et al.</i> (53)	2010	22	> 35%	Post-reperfusion and organ procurement core needle biopsy. H&E and frozen sections included. MaS: Definition not provided.	DBD	330	5-year survival 74.4%; Prolonged hospitalisation, more frequent transfusion of hemocomponents and higher peak of transaminases
Li <i>et al.</i> (54)	2009	23	21–40% (excluded if > 60%)	Post-reperfusion and during hospitalisation core needle biopsies. H&E on frozen sections. MaS: Definition not provided.	DBD	355	1-year survival 87%; 21.7% early graft dysfunction and prolonged hospitalisation
Frongillo <i>et al.</i> (52)	2009	24	16–30%	Bench wedge and core needle biopsies. H&E and oil red O staining. MaS: single vacuole larger than and displacing the nucleus of the cell. Pathologist plus digital analysis of steatosis	DBD	487	6-month graft survival 80%; Primary dysfunction was 12.5%, and 8.4% primary non-function
Angele <i>et al.</i> (50)	2008	50	> 30%	Post-reperfusion core needle and wedge biopsies. H&E staining. MaS: single vacuole larger than and displacing the nucleus of the cell.	DBD	Not reported	5-year survival 58%; 4 cases primary non-function; Higher peak of transaminases
Nikeghbalian <i>et al.</i> (55)	2007	34	30–60% (excluded if > 60%)	Not specified timing or biopsy type. H&E staining. MaS: Definition not provided, and assessment performed semiquantitatively in 100 cells.	DBD	Not reported	1-year survival 73%; No mention to delayed graft function or primary non-function

McCormack <i>et al.</i> (63)	2007	6	30–60%	Core needle biopsy during organ procurement. H&E staining. MaS: Definition not provided.	DBD	562	1-year survival 100%; 0% primary non-function rate
Briceño <i>et al.</i> (56)	2005	67	30–60%	Post-reperfusion and organ procurement core needle biopsy. H&E staining. MaS: lipid droplets displacing the hepatocyte nucleus and occupying the majority of the cytosol.	DBD	166	90-day graft survival 72%; 1.5% primary non-function and 7.5% delayed non-function
Verran <i>et al.</i> (57)	2003	48	30–60%	Post-reperfusion biopsy (not specified type). Staining used not mentioned. MaS: Definition not provided.	DBD	Not reported	3-month survival 78%; 35% poor initial graft function, 2% primary non-function
Zamboni <i>et al.</i> (58)	2001	8	> 25%	Not specified timing or type of biopsy. H&E staining. MaS: Definition not provided.	DBD	Not reported	Overall shorter patient survival; 62.5% delayed non-function and 12.5% primary non-function
Canelo <i>et al.</i> (59)	1999	14	30–60%	Biopsy taken during transplantation and type not specified. Staining used not mentioned. MaS: definition not provided.	DBD	Not reported	65% initial dysfunction and 14% initial nonfunction

**Abbreviations:** MaS- Macrovesicular steatosis; DBD: Donation after brain death; DCD: Donation after circulatory death; CIT: cold ischaemia time.

#### 5.4.1.7 Steatosis and organ donation

In the literature, the prevalence of steatosis in donor organs range from 13% to 28% (66). Steatosis was the reason for 42% of donor livers being declined for transplantation in Spain in 2000 (67). The National Health Service annual report on liver transplantation revealed that in 2016, in the United Kingdom, 16.6% of the total 1001 livers retrieved in the period of 2015–2016 were not transplanted (12). More importantly, steatosis was the main reason for declining the liver in 39% of cases (12). Hence reducing liver steatosis and recovering its function with MP would potentially allow a significant expansion of the donor pool.



#### **5.4.2 *Ex situ* machine perfusion of donor livers**

*Ex situ* MP of donor livers has been demonstrated to be a safe intervention and may improve the liver quality (10, 68). This beneficial effect may be more apparent in high-risk ECD organs which are more susceptible to ischaemic injury during cold preservation (9). The two most explored techniques of MP are briefly discussed below.

Hypothermic machine perfusion is performed at temperatures close to 10 °C using a dual vessel perfusion (hepatic artery and portal vein) model or a single vessel perfusion model through the portal vein alone. The perfusate oxygenation may be derived from a non-oxygenated perfusion scheme (69) to a highly oxygenated hypothermic perfusion set-up through the portal vein (HOPE) alone (70). HOPE was shown in experimental models to improve mitochondrial function decreasing the production of ROS and reducing the activation of the inflammatory cascade (71, 72).

Normothermic machine perfusion of the liver (NMP) is performed at 37 °C. Its main benefit is its ability to maintain the organ in a near-physiological environment at full metabolism. This allows the assessment of metabolic parameters for liver viability prior to transplantation (68). Moreover, it also provides a window of opportunity for *ex situ* pharmacological interventions during preservation with the aim of organ reconditioning.

##### **5.4.2.1 *Ex situ* machine perfusion of steatotic donor livers**

There is limited data on the use of MP in steatotic donor livers. Defatting of steatotic livers is an intuitive idea to allow the utilisation of these organs for transplantation. Defatting strategies should not only aim to reduce the triglyceride load in steatotic hepatocytes but attempt to improve the organ metabolism and lessen the impact of IRI. Defatting would be achieved

enhancing the lipid metabolism, increasing the cellular exportation of intracellular triglyceride as Very Low-Density Lipoprotein (VLDL) and the fatty acid mitochondrial  $\beta$ -oxidation (73).

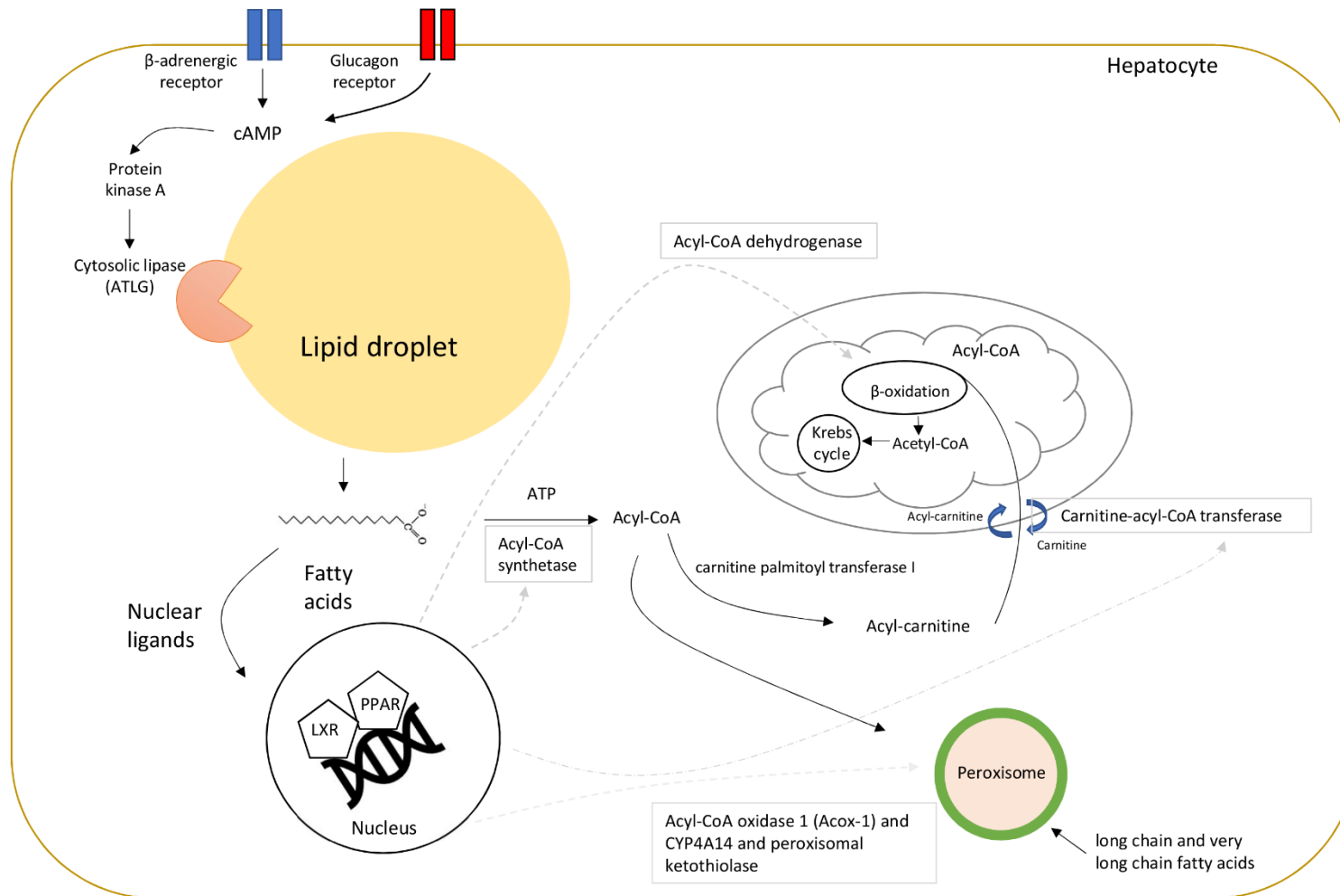
#### **5.4.2.2 Potential targets for intervention in the lipid metabolism during NMP**

Therapeutic targets to manipulate the lipid metabolism during NMP involve mainly the modulation of the production of enzymes involved with oxidation of FA for energy production and/ or FA cellular exportation. As a consequence, cytoplasmic lipases play a key role as initiators in the mobilisation of intracellular triacylglycerol stores and conversion into FAs and glycerol (74). Between those lipases the adipose triglyceride lipase (ATGL) is considered to be the limiting rate factor for intracellular lipolysis in hepatocytes (75–77). The FAs released into the cytosol apart from being a substrate for metabolism also act as a ligand for nuclear receptors (NR) promoting the transcription of enzymes related to the catabolism and exportation of FAs (78, 79). In addition, pharmacological interventions can also activate the expression of those NRs and hence modulate the intracellular catabolism of FAs (75, 79). Peroxisome proliferator-activated receptors (PPAR) are a class of NRs that, when activated, promote transcription of hepatic mRNAs related to mitochondrial  $\beta$ -oxidation of FA, such as acyl-CoA oxidase, carnitine palmitoyltransferase-1, liver-fatty acid binding protein and peroxisomal ketothiolase (80, 81). The delta-isoform of PPAR is also associated with a concomitant drop in inflammatory marker levels, namely transforming growth factor-beta 1, IL-6, monocyte chemoattractant protein 1, tumour necrosis factor, alpha and nuclear factor kappa B 1 (82). The  $\alpha$ -isoform of PPAR has been shown to decrease serum triglycerides levels, as fenofibrate (83). Moreover, it was shown in obese animal models that PPAR- $\alpha$  transcription levels are increased during regular exercise in comparison with sedentary animals (81).

Another NR associated with lipid metabolism is the pregnane X receptor (PXR). PXR is a NR involved in the cellular elimination of toxic drugs and xenobiotics (84). Transgenic PXR-knock-out mice were shown to have a reduction in the acyl-CoA oxidase 1 (Acox-1) mRNA levels. This enzyme is responsible for the peroxisomal  $\beta$ -oxidation of very long chain FA and concomitantly higher protein levels of the gene CYP4A14. In turn, CYP4A14 converts long chain FA to toxic dicarboxylic acids that will be used for peroxisomal  $\beta$ -oxidation. Dicarboxylic acid, which accumulates as a consequence of reduced ACOX-1 peroxisomal  $\beta$ -oxidation, is implicated in mitochondrial damage and steatosis (85).

Hormones are also involved in the modulation of the lipid metabolism. The stimulation of  $\beta$ -adrenergic receptors on hepatocytes activates via the cyclic adenosine monophosphate (cAMP) the protein kinase A (PKA) recruiting cytoplasmic lipases as ATGL to the surface of the lipid droplets initiating the process of lipolysis. Glucagon binding to its receptor also acts via the cAMP-PKA pathway to promote lipolysis on hepatocytes. Glucagon was shown to reverse hepatic steatosis in rats that receive the hormone in parenteral nutrition (86). Visfatin or pre-B-cell colony-enhancing factor 1 is an adipokine increased in obesity. It has insulin mimetic effects in many tissues and enhances the nicotinamide phosphoribosyltransferase (Nampt) activity in the nicotinamide adenine dinucleotide (NAD<sup>+</sup>) pathway and leads to a decrease the glucose serum levels (87, 88). The exact mechanism linking it to lipid metabolism in steatotic livers is not clear yet (89). These signalling pathways maybe targeted by pharmacological and non-pharmacological methods and these strategies are discussed below. Figure 5.4 illustrates the interaction between cytoplasmic lipases, hormones and lipolysis within hepatocytes.

**Figure 5.4: Potential cellular targets for defatting therapies.**



**Figure 5.4: Potential cellular targets for defatting therapies.** Stimulation of  $\beta$ -adrenergic and/or glucagon membrane receptors activates the cyclic adenosine monophosphate (cAMP) - protein kinase A (PKA) pathway that in turn recruits cytoplasmic lipases as the adipose triglyceride lipase (ATGL) to the surface of the lipid droplets. Fatty acids and glycerol will be released into the cell, as a result of the intracellular lipolysis, serving not only as substrates for the cell metabolism but also as ligands to nuclear receptor (peroxisome proliferator receptor [PPAR] and liver X receptors [LXR]) increasing the transcription of enzymes involved in the catabolism of fatty acids in the mitochondria and peroxisome. The squares contain the specific enzymes that have an upregulation in the transcription as a consequence of this stimulus. Cytosolic fatty acid reacts with adenosine triphosphate (ATP) molecules producing fatty acyl adenylate, which in turn react with free coenzyme A originating fatty acyl-CoA. Carnitine palmitoyltransferase I catalyse the reaction of acyl-CoA with the hydroxyl group of carnitine by carnitine palmitoyltransferase. Acyl-carnitine is transported inside the mitochondria by a Carnitine-acyl-CoA transferase and a carnitine is transferred outside. Acyl-CoA is processed by  $\beta$ -oxidation. Alternately, long and very-long-chain fatty acids are also oxidized in the peroxisomes.

## 5.4.3 Defatting Strategies

### 5.4.3.1 Non-pharmacological interventions

NMP alone without the administration of additional therapy has been associated with hepatocyte defatting in animal models (90, 91). Jamieson *et al.* 2011, using a porcine model, showed that NMP decreases hepatic steatosis over periods of 24 and 48 hours. Moreover, NMP increased urea production and sustained factor V and bile production with expression of markers for reperfusion injury comparable to normal livers (91). Nagrath *et al.* 2009 reported a 30% reduction in triglycerides content in rats' liver over a 3 hours' period of NMP (90).

One recent published study with discarded human livers presenting different degrees of steatosis showed that NMP alone for up to 24 hours can increase the lipid content in the perfusate, however it has not found any histological change in the fatty content of the organs (92). The authors discuss the divergent findings from animal models associating it with differences across species and the process of chronic fat accumulation in human livers (92).

### 5.4.3.2 Pharmacological interventions

In 1989 Pégrier *et al.*, demonstrated that glucagon, dibutyryl cyclic AMP and forskolin induced ketogenesis in newborn rabbit hepatocytes using sodium oleate as a substrate in a L-carnitine supplemented medium (93). Forskolin and glucagon modulate adenylate cyclase and stimulate production of the second messenger cyclic AMP. cAMP is a secondary messenger, acting as a signal transducer as it is instrumental in providing a link across the cell membrane between extracellular hormone molecules and intracellular processes (94).

The androstane receptor ligand Scorparone (6,7-dimethoxycoumarin) was shown to improve the viability of hepatocytes preserved in Euro-Collins solution, with lower levels of malondialdehyde and alanine aminotransferase (ALT) found in the supernatant (95). It also has vasodilatory properties, enhancing the production of prostacyclin which blocks platelet aggregation and possesses oxygen radical's scavenger-like properties (96). Scorparone, used with other drugs into a cocktail, is associated with reversal of hepatocyte steatosis through the higher production of adiponectin and upregulation of PPAR- $\gamma$  (97).

#### **5.4.3.2.1 The defatting cocktail**

In 2009, Nagrah *et al.* reported that a combination of PPAR $\alpha$  ligand GW7647 and GW501516, PXR ligand Hypericin, the constitutive androstane receptor ligand Scorparone, the glucagon mimetic cAMP activator forskolin, and the insulin-mimetic adipokine visfatin caused a reduction in intracellular lipid content of 35% within 48 hours in rat hepatocytes which were made steatotic by FA supplementation in static culture (90). Furthermore, this cocktail of drugs at the same concentration was added to the perfusion fluid in a model of NMP of whole fatty rat livers. This model accelerated the process of defatting and a simultaneous reduction of 50% in intracellular triglycerides levels within 3 hours. Interestingly, a reduction in 30% intracellular triglyceride was observed to have occurred even in the control perfusion group without the

combination of drugs in the perfusion fluid. Histological analysis of the tissue revealed a lower amount of intracellular lipid droplets in the peri-portal areas. The authors correlated this reversal in steatosis with increased mRNA levels of triacylglycerol hydrolase (an enzyme that promotes triglyceride hydrolysis in hepatocytes), apolipoprotein B-100, carnitine palmitoyltransferase I and acyl coenzyme A oxidase. The acceleration in the defatting process was considered to be related to greater oxygen availability and therefore increased beta-oxidation rate (90).

Following this study, Nativ *et al.* employed a rat hepatocyte cell culture model to investigate the impact of this cocktail of drugs during IRI. They noted enhanced exportation of intracellular triglycerides and increased ketone body secretion. The last observation was considered to be consequence of augmented mitochondrial oxidation of FAs by improved acetyl coenzyme A delivery to the mitochondria. In addition, they reported a 32% reduction in ROS production, 27% increase in ATP levels and high cell viability with lower lactate dehydrogenase (LDH) release. This suggested improved resistance of the treated cells to IRI (98).

The same protocol was later applied to human hepatoma cells (HepG2 cells) by Yarmush *et al.* (99). They found that the cells' lipid content decreased if left in an environment free of FAs. Analysis of metabolites present in the culture media showed that this defatting process was attributed to increased mitochondrial  $\beta$ -oxidation through upregulation of the tricarboxylic acid and urea cycles. Furthermore, the defatting process was accelerated when exposed to the defatting combination of drugs and hyperoxic conditions in comparison with the cells that received only the cocktail (99). Thereafter, the same author used HepG2 cells to study the impact of flow on the defatting process in an *in vitro* model using a bioreactor where the cells were kept under constant flow with controlled shear stress force (100). Similar defatting rates were observed using the cocktail over 6 hours under flow conditions and 48 hours under static conditions. They concluded that the transport of L-carnitine, which supplies free fatty acids to

the mitochondria, is a key factor in the modulation of defatting under flow conditions and flow rates should be adequate enough to ensure that this is delivered to the cells (100).

Liu *et al.* 2013 attempted to defat rat steatotic livers using the cocktail at midthermic temperatures (101). Steatotic livers were perfused using a model of MP at 20 °C for 6 hours. VLDL and triglyceride concentrations increased in the perfusate of vehicle and drug group. However, no significant changes were observed in histology and intracellular triglyceride content at 20 °C. It was concluded that defatting is a process dependent on physiological temperatures (with the organ's metabolism in full swing) to be effective (101).

#### **5.4.3.2.2 Glial cell line–derived neurotrophic factor**

Another pharmacological defatting intervention reported in the literature is the use of glial cell line–derived neurotrophic factor (GDNF). This growth factor is involved in the development of enteric nervous cells and it has been shown to optimise glucose control in rats by promoting pancreatic  $\beta$ -cell proliferation (102). GDNF transgenic mice, which overexpress GDNF, were fed a fatty rich diet and exhibited resistance to weight gain, diminished insulin resistance, dyslipidaemia, hyperleptinemia, and less hepatic steatosis (103). Vakili *et al.* 2016 used this agent in an *in vitro* model of HepG2 cells which were made fatty by supplementation with palmitic acid. They exposed the fatty cells to 48 hours of incubation with 100 ng/mL GDNF and observed a reduction in intracellular triglyceride content, reaching levels similar to lean cells. However, it should be highlighted that these cells are not primary human hepatocytes and this response could be different. Furthermore, using a model of NMP, the authors perfused 4 fatty mice livers for 4 hours with GDNF in the perfusate and reported an effective reduction of 40% in intracellular lipid content. Another interesting point of this study was that fatty livers were also perfused using the defatting combination of drugs described by Nagrath *et al.* in 2009



and showed that GDNF was effective in clearing lipid droplets, decreasing intracellular triglyceride levels and leaving less LDH in the perfusate (104). Hence GDNF may be a promising option for defatting steatotic livers although the latter study did not include histological analysis of livers subjected to defatting strategies.

Large animal models of steatosis (e.g. pigs) would potentially be more reliable for further investigations as they present metabolic functions closer to human livers (105). However, despite one study reporting successful induction of steatosis in pigs using a protein deficient diet, to our knowledge, no other studies have explored or reported on similar models (105).

#### **5.4.3.2.3 Considerations for human livers**

Banan *et al.* 2016 supplemented the perfusate of two human livers during NMP with 10 mM of L-carnitine to enhance mitochondrial oxidation of FAs. Over 8 hours of perfusion a decrease of 10% in MaS was reported (106). From the two organs, one with 30% MaS had minimal changes over time and the other one with 80% MaS pre-perfusion exhibit a 10% reduction in large droplet MaS (106). This variability between the two organs reported and the lack of a control group dampen enthusiasm for these preliminary results.

The results reported by studies in pharmacologically-induced *ex situ* defatting of steatotic livers using MP have limitations. First and foremost, all studies properly designed to study this subject up to this point in the literature involved rat models as or *in vitro* work with tumour cells. Consequently, it is still not explored how whole human livers would respond to these specific interventions. Secondly, steatotic livers in these models were perfused at normothermic temperatures immediately after retrieval. Due to the logistics involved with current methods of organ preservation and transportation, donor livers still currently subjected to a cold ischaemic preservation period. In this scenario whether the beneficial effect seen would be the same for a

whole human liver is not known. Furthermore, it remains to be seen whether any benefit is sustained after the liver is removed from the machine. The extrapolation of results of the ideal concentration of drugs from animal models to human livers is also a challenging issue. In addition, individual effect of some of drugs included into the defatting cocktail on lipid metabolism (e.g. scorparone and visfatin) still not fully characterized. Theoretical benefits of using filters to either remove solubilised lipids from the perfusate or prevent lipotoxicity still needs to be proven.

In terms of time required for defatting we would ideally envisage periods of few hours when concomitant assessment of metabolic parameters could offer an indication of organ viability before transplantation. However apart from animal studies data suggesting that it is achievable within 3 hours, there is no data in the literature to support assertions in human livers and at this time conclusions remain speculative (90). Models of discarded steatotic donor human livers are likely to provide valuable insights into these outstanding questions however only transplantation would offer a definitive answer as criteria for organ viability during NMP still under investigation (107). Table 5.2 summarizes the drugs used in experimental models for defatting and the pathway involved.

Spontaneous reversal of steatosis after LT has been described (53, 60, 63). Westerkamp *et al.* 2015 reported a decrease from 30–60% to  $\leq 10\%$  within a median of 12 days (60). Considering the mechanisms of defatting explored in this review, we hypothesize that the stress response to surgery with the release of glucagon and catecholamines may enhance lipolysis and promote defatting *in vivo*.

**Table 5.2: Drugs employed in experimental models for *ex vivo* defatting of steatotic livers and its metabolic pathway involved.**

Drugs		Defatting pathway		Additional benefits
<b>Defatting cocktail</b>	<b>Peroxisome proliferator-activated receptors (PPAR) GW7647 and GW501516</b>	Promote transcription of hepatic mRNAs related to mitochondrial $\beta$ -oxidation of fatty acids, such as acyl-CoA oxidase, carnitine palmitoyltransferase-1, liver-fatty acid binding protein and peroxisomal ketothiolase (80, 81).	The cocktail of drugs increased mRNA levels of triacylglycerol hydrolase, apolipoprotein B-100, carnitine palmitoyltransferase I and acyl coenzyme A oxidase (90).	The delta-isoform of PPAR is also associated with a concomitant drop in inflammatory marker levels, namely transforming growth factor-beta 1, IL-6, monocyte chemoattractant protein 1, tumor necrosis factor, alpha and nuclear factor kappa B 1 (82).
	<b>Pregnane X receptor (PXR) ligand Hypericin</b>	Increased peroxisomal $\beta$ -oxidation of very long chain fatty acids via higher acyl-CoA oxidase 1 (Acox-1) mRNA levels and concomitantly higher protein levels of the gene CYP4A14. In turn, CYP4A14 converts long chain fatty acids to toxic dicarboxylic acids that will be used for peroxisomal $\beta$ -oxidation (84, 85).		
	<b>Constitutive androstane receptor ligand Scorparone</b>	Upregulation of PPAR- $\gamma$ * (97).		Vasodilatory properties, enhancing the production of prostacyclin which blocks platelet aggregation and possesses oxygen radical's scavenger-like properties (96).
	<b>Glucagon mimetic cAMP</b>	Increased lipolysis of lipid droplets in hepatocytes and		

	<b>activator forskolin</b>	cellular oxidation of fatty acids (94).		
	<b>Insulin-mimetic adipokine visfatin</b>	Not established (89).		Enhances nicotinamide phosphoribosyltransferase (Nampt) activity in the nicotinamide adenine dinucleotide (NAD+) pathway and leads to a decrease the glucose serum levels (87, 88).
<b>Glial cell line–derived neurotrophic factor (GDNF)</b>		Peroxisome proliferator activated receptor- $\alpha$ and $\beta_1$ - and $\beta_3$ -adrenergic receptor genes, which are associated with increased lipolysis and enhanced lipid $\beta$ -oxidation (103, 104).		

\*Effect reported for Scorparone as one of the active compounds of a cocktail of Chinese herbs.

#### 5.4.4 Other potential strategies to rescue steatotic organs

The main target for the use of MP in steatotic livers nevertheless should not be only defatting, instead MP should rescue their metabolic parameters into limits considered safe for transplantation. Preconditioning of steatotic livers using HMP or midthermic machine perfusions, may improve their metabolic activity and enable them to negate IRI (108).

##### 5.4.4.1 Hypothermic perfusion and steatotic livers

Bessemis *et al.* 2007, using a murine model reported that hypothermically perfused steatotic livers had a higher rate of bile production, ammonia clearance, urea production, oxygen consumption and recovery of ATP levels in comparison with SCS (109). Kron *et al.* 2017, in a transplant rat model of severely steatotic livers submitted to 12 hours of SCS showed that short term HOPE prior to implantation was able to diminish IRI assessed by oxidative injury, nuclear damage, Kupffer and endothelial cell activation without changing the steatosis grading of the

organs (110). The same study reported results of six moderately steatotic human livers that had HOPE prior to transplantation suggesting achievement of similar benefits. However, some observations deserve attention as HMP period was variable (1.8–4.8 hours), livers had short CIT (median 4.1 hours) and MaS rates differ slightly from controls (HOPE 20–40% vs. controls 25–60%).

Conversely, one study performing non-oxygenated HMP for 24 hours on human livers discarded mainly for steatosis reported impaired ATP recovery rate, increasing perfusate lactate levels over time with significant differences in release of markers of hepatocellular injury in the perfusate (AST and LDH) between steatotic livers and ‘transplantable’ discarded livers (111). In contrast to the former non-transplantable steatotic organs presented with irreversible mitochondrial damage on electron microscopy at the end of 24 hours perfusion (111).

#### **5.4.4.2 Midthermic MP and steatotic livers**

Midthermic MP at 20 °C was shown in experimental rat models to offer beneficial preservation for steatotic organs in comparison with SCS by potentially optimising energy status (112). It was also suggested to play a protective role in IRI by decreasing TNF- $\alpha$ , caspase-3 activity thus being associated with lower levels of oxidative injury and damage to the intrahepatic biliary tree (113–115). Conversely, another study examining the metabolic profiles of discarded human livers perfused for 3 hours at 20 °C, steatotic livers were found to show higher expression of markers of injury, impaired ATP recovery rate and higher hepatic artery flow resistance (116). Importantly these studies were performed in non-transplant models and further highlight the differences between human livers and current animal models.

#### **5.4.4.3 Other strategies to lessen ischaemia-reperfusion injury**

Pharmacological strategies to lessen the impact of organ injury during reperfusion can also be a means of overcoming the risks associated with steatotic livers. A prominent example is the proposed use of the antibiotics minocycline and the cyclophilin inhibitor NIM811. These have been shown, in a rodent model to promote preservation of mitochondrial polarization and lower activation of apoptosis/ necrosis pathways with higher graft survival rates (117). NIM811 has also associated with better liver regeneration after extended hepatectomy, blocking ATP depletion and activation of cell apoptosis (118). Marshall *et al.* 2014 studied the inhibition of the terminal membrane attack complex using a site-targeted murine complement inhibitor (CR2-CD59) to inhibit the complement pathway in the context of IRI. CR2-CD59 enhanced cellular proliferation/regeneration after extended liver resection and improved survival rates from 0 to 70% (119).

Recently He *et al.* 2018 described the first case of ischemia-free organ transplantation (IFOT) for a severely steatotic donor liver (120). The authors reported that the recipient did not have post-reperfusion syndrome, and the organ demonstrated minimal signs of hepatobiliary injury and reduced pro-inflammatory response (120). IFOT might be a promising approach to decrease IRI increasing not only the utilisation, but also reducing complications associated with ECD organ transplantation. Logistically mobile preservation devices would allow ‘rollout’ of this strategy from in-house to inter-hospital. In terms of steatotic organs, it highlights that strategies to ameliorate IRI can improve the outcomes of such organs post-transplant.

The use of NMP in steatotic livers immediately after retrieval as a preservation technique would eliminate additional cold injury, avoiding intense depletion of ATP and offering additional time for defatting procedures and viability assessment. Other interventions associated with a reduction in post-transplant complications of steatotic livers have been described. In the context of living donation, donors supplemented with  $\Omega$ -3 fatty acids one month before organ

procurement suffered less postoperative complications. Although the results are promising, the therapy cannot be applied to deceased donors (51, 121).

## **5.5 Discussion**

In this review we described how the cellular changes induced by steatosis could translate into higher incidence of post-transplant complications. The evidence for the clinical use of these organs and the current ‘other risk factor minimisation strategy’ was briefly explored. However, despite this approach being already known by transplant surgeons, steatosis remains a major discarding reason for livers worldwide (12). MP has a potential role to increase organ utilisation by offering opportunities for organ reconditioning and functional assessment (9, 11). Therefore, the use of this device for preconditioning steatotic donor livers deserves urgent attention. The current evidence suggests that the key point for its safe utilisation is avoidance of detrimental effects of IRI. Defatting involves pharmacological modulation of intracellular metabolic pathways. Enhancing lipolysis intracellular triglycerides stores serve as the substrate for energy production and ligands for nuclear receptors optimizing mitochondrial function. It also leads to lower production of ROS, release of markers of cellular injury and improved cell viability (90, 104). Moreover, there is the potential to improve the hepatic microcirculation, lower activation of Kupffer cells and decrease release of inflammatory mediators during reperfusion, resulting in less subsequent tissue injury. Finally, improvements in organ functioning may potentially be observed during NMP (107).

At present the studies investigating defatting strategies have focused heavily upon animal models and the extrapolation of these to the clinical setting is in need of urgent investigation. The same applies to steatotic livers and MP where only few studies are available. Data from clinical series of transplantation of steatotic livers are highly heterogeneous. Hence although

we have used a comprehensive search strategy only few studies addressed this subject, therefore the risk of bias within the literature needs to be borne in mind.

Other strategies described have also the potential to limit the detrimental impact of IRI on highly vulnerable steatotic donor livers. The ideal method of MP to improve their utilisation still requires definition as comparative clinical data is lacking. This highlights the major limitation of the present review. In addition, it is difficult to make firm conclusions regarding steatotic organ utilisation once definition of MaS, technique for liver biopsy and steatosis assessment is not standardised between studies.

## **5.6 Conclusion**

Steatotic livers pose a conundrum in liver transplantation. These organs sustain greater injury but form an ever increasing proportion of the donor pool. Although some series report successful long-term outcome of patients transplanted with steatotic livers these are limited. MP provides a window of opportunity to improve the quality of steatotic donor livers by acting as a vehicle for the application of pharmacological and non-pharmacological interventions. Defatting of donor livers in MP is a promising approach to organ reconditioning. However, further research is required into this field before it can be considered a plausible and feasible clinical option.



## 5.7 Appendices

### 5.7.1 Table S5.1: Literature search strategy (example for one database).

Database	Strategy
PubMed	'machine perfusion' (MeSH) AND 'liver transplantation' (MeSH) AND 'steatosis' (text) OR 'fatty livers' (text) OR 'defatting' (text) OR 'steatosis reversal' (text)

**Abbreviations:** MeSH: Medical subject heading; text: Text word

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## Chapter 6

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# AN EFFECTIVE PROTOCOL FOR PHARMACOLOGICAL DEFATTING OF PRIMARY HUMAN HEPATOCYTES WHICH IS NON-TOXIC TO CHOLANGIOCYTES OR INTRAHEPATIC ENDOTHELIAL CELLS

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### Published article

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Y.L.B. designed the experiment, performed the experiment, interpreted the data and wrote the manuscript. L.W. assisted in the technicalities of performing the experiments. A.P.C.S.B. assisted in some of the experiments. D.F.M. and H.M. offered surgical support for the experiments. R.H.B. assisted in editing the manuscript. S.A. designed the experiments, assisted in the interpretation of the data and editing the manuscript.

## 6.1 Abstract

**Introduction:** Pharmacological defatting of rat hepatocytes and hepatoma cell lines suggests that the same method could be used to ameliorate macrovesicular steatosis in moderate to severely fatty livers. However, there is no data assessing the effects of those drugs on primary human liver cells. We aimed to determine the effectiveness of a pharmacological cocktail in reducing the *in vitro* lipid content of primary human hepatocytes (PHH). In addition, we sought to determine the cytotoxicity of the cocktail towards non-parenchymal liver cells.

**Methods:** Steatosis was induced in PHH by supplementation with a combination of saturated and unsaturated free fatty acids. This was followed by addition of a defatting drug cocktail for up to 48 hours. The same experimental method was used with human intrahepatic endothelial cells (HIEC) and human cholangiocytes. MTT assay was used to assess cell viability, triglyceride quantification and oil red O staining were used to determine intracellular lipids content whilst ketone bodies were measured in the supernatants following experimentation.

**Results:** Incubation of fat loaded PHH with the drugs over 48 hours reduced the intracellular lipid area by 54%, from 12.85% to 5.99% ( $p = 0.002$ ) (percentage of total oil red O area), and intracellular triglyceride by 35%, from 28.24 to 18.30 nmol/million of cells ( $p < 0.001$ ). Total supernatant ketone bodies increased 1.4-fold over 48 hours in the defatted PHH compared with vehicle controls ( $p = 0.002$ ). Moreover, incubation with the drugs for 48 hours increased the viability of PHH by 11%, cholangiocytes by 25% whilst having no cytotoxic effects on HIEC.

**Conclusion:** These data demonstrate that pharmacological intervention can significantly decrease intracellular lipid content of PHH, increase fatty acids  $\beta$ -oxidation whilst being non-toxic to PHH, HIEC or cholangiocytes.

## 6.2 Background

Hepatic steatosis results from the accumulation of triacylglycerol in the cytoplasm of hepatocytes which coalesce to form lipid droplets (LD). Large LDs that cause displacement of the cell nucleus are termed macrovesicular steatosis. Donor livers with macrovesicular steatosis are associated with significantly increased risk of early graft dysfunction after liver transplantation (1–4). Intuitively defatting of steatotic donor livers could potentially improve both the organ utilisation and patient outcomes after transplantation. Using a static *in vitro* rat hepatocyte model where cells were loaded with fat, Nagrath *et al.* reported a reduction of 35% in the intracellular lipid content over 48 hours by supplementing media with a defatting cocktail consisting of peroxisome proliferator-activated receptor (PPAR) $\alpha$  ligands GW7647 and GW501516, pregnane X receptor (PXR) ligand Hypericin, the constitutive androstane receptor (CAR) ligand Scorparone, the glucagon mimetic cyclic adenosine monophosphate (cAMP) activator forskolin and the insulin-mimetic adipokine visfatin (5). Thereafter the defatting cocktail was used in a model of *ex situ* normothermic machine perfusion (NMP) of a whole steatotic rat liver and a reduction of 50% in the intracellular triglycerides levels was observed within 3 hours (5). Subsequently the same protocol was applied to human hepatoma cells (HepG2 cells) by Yarmush *et al.* and similar findings were reported (6). Consistently these studies demonstrate that the defatting cocktail increased mitochondrial beta-oxidation of fatty acids (FA) as represented by higher production of ketone bodies and upregulated the transcription of key enzymes involved with the exportation of intracellular lipids and oxidation of FA in the peroxisome (5, 6).

However, HepG2 cells do not accurately represent the response of primary human hepatocytes (PHH) to drugs or cellular stresses such as hypoxia and hypoxia/reoxygenation (7–9). In particular HepG2 cells demonstrate a 90% reduction in cytochrome P450 expression (10). Therefore, before such defatting strategies can be considered for the use in machine perfusion

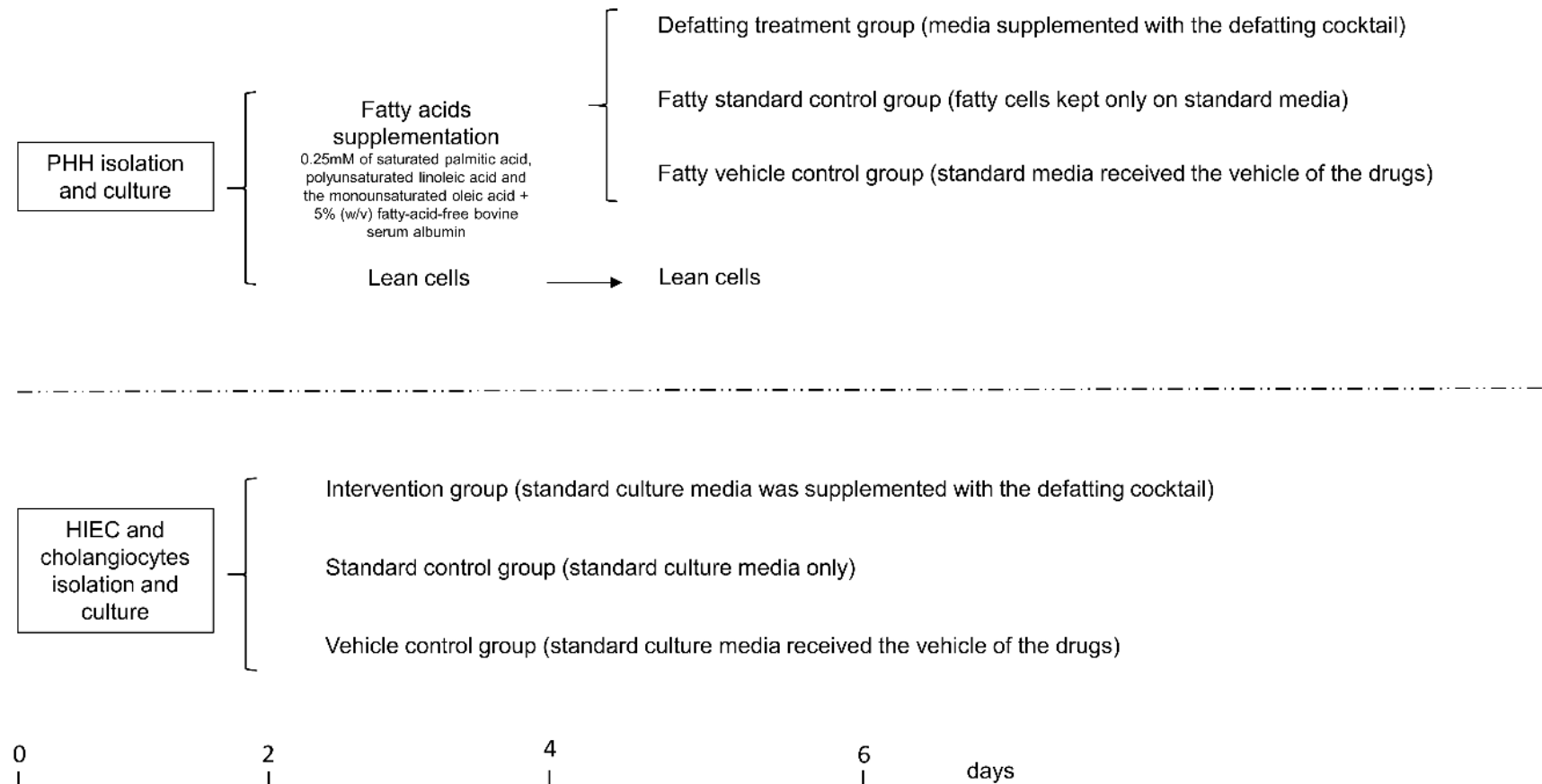
of human donor livers, it is imperative that their efficacy and cytotoxicity be determined in models using human liver cells (11, 12). In particular the cytotoxicity of the defatting drugs on other cell types within the liver, such as intrahepatic endothelial cells (HIEC) and cholangiocytes, has not been assessed. The aim of the present study was to investigate the efficacy of this defatting drug cocktail on steatotic PHH and its cytotoxicity towards PHH, HIEC and cholangiocytes.

## **6.3 Methods**

### **6.3.1 Study design**

Steatosis was induced in PHH by incubation of cells with FAs. Fatty loaded PHH were then incubated with a cocktail of defatting agents to test its cytotoxicity and effectiveness in reducing the intracellular lipid content. HIEC and cholangiocytes were also incubated with the defatting cocktail for 48 hours to assess the cytotoxicity of the cocktail. Three separate experiments were performed in quadruplicate. Figure 6.1 shows a schematic view of the study design.

**Figure 6.1: Study design.**



**Figure 6.1: Study design.** Series 1: Isolated primary human hepatocytes (PHH) were left in standard media for 2 days and then received media supplemented with fatty acids. After 2 days of fat loading, the fatty PHH were allocated to the defatting treatment group where the media was supplemented with the defatting cocktail of drugs, and the control groups, the standard control group and the vehicle control group that received vehicle only. Lean hepatocytes were kept in standard culture conditions throughout the experimental period. The experimentation period lasted for two days thereafter. Series 2: Human intrahepatic endothelial cells (HIEC) and cholangiocytes were immuno-magnetically separated with Dynabeads conjugated with cell-specific monoclonal antibody. The cells were left in culture for 2 days in standard media to reach confluence and then were allocated to the intervention group that received the defatting cocktail and the control groups, the standard control group and the vehicle control group that had the media supplemented with the vehicle only. The experimentation period lasts for two days thereafter.

### **6.3.2 Source of Liver Tissues**

The human cells were isolated from discarded donor livers. The organs were initially offered, accepted, and procured with the intention of clinical transplantation. They were then declined by all UK transplant centres and offered for research by the National Health Service Blood and Transplant (NHSBT) coordinating office. Specialist nurses in organ donation obtained consent to use donor tissue for research as part of the consent process for standard clinical organ donation. None of the donor organs were from a vulnerable population and all next of kin provided written informed consent that was freely given. Ethical approval for the study was granted by the London-Surrey Borders National Research Ethics Service committee as well as Loco-Regional and NHSBT Ethics Committees (reference 13/LO/1928 and 06/Q702/61). Cells were isolated from three donors after brain stem death declined for transplantation because of logistics and were preserved by static cold storage in University of Wisconsin preservation fluid.

### **6.3.3 PHH cell isolation**

A published collagenase perfusion technique was employed for PHH isolation from liver wedges (13). Briefly liver was digested and centrifuged to isolate PHH. These cells were

resuspended and then plated on 24-well plates previously coated with rat tail collagen type 1 at a density of  $3 \times 10^5$  cells/well in Dulbecco's Modified Eagle's Medium (DMEM) (Catalogue number [CN]:41965-039; Gibco laboratories, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) (CN:10270106; Gibco) and 5% glutamine/penicillin/ streptomycin (GPS) (CN:10378016; Gibco). After 2 hours the cells were washed with Phosphate-buffered saline (PBS) (CN:10010023; Gibco) and the media changed to our standard medium for PHH culture, constituted of Arginine-/Glutamine-free Williams E (CN:12551032; Gibco) with 1% GPS, hydrocortisone (2  $\mu$ g/ml) (H4001; Sigma-Aldrich, St. Louis, MO., USA), insulin (0.124 U/ml) (I2643; Sigma-Aldrich) and L-ornithine (400  $\mu$ M) (O6503; Sigma-Aldrich), subsequently the cells were kept at 37 °C in 95% air/ 5% CO<sub>2</sub>.

#### **6.3.4 *In vitro* steatosis induction for PHH**

The standard media for PHH culture was supplemented with a combination of FAs in order to promote increases in the intracellular triglyceride levels stocked as LDs, as previously described (14). This fattening media consisted of the saturated palmitic acid (P0500; Sigma-Aldrich), polyunsaturated omega-6 linoleic acid (L5900; Sigma-Aldrich) and the monounsaturated omega-9 oleic acid (O1257; Sigma-Aldrich) all at a final concentration of 0.25 mM. This concentration was determined by performing cytotoxicity titration experiments prior to institution of the full experimental protocol. A supplement of 5% fatty-acid-free bovine serum albumin weight/volume (BSA) (A3803; Sigma-Aldrich) was added as a protein carrier. The media was changed daily, and the steatosis induction period was 48 hours. The lean control group was incubated with standard media only throughout the experimental period.

#### **6.3.5 Defatting medium for PHH**



Following steatosis induction, the fattening media was removed, and cells washed with PBS. Experiments were then performed on 4 distinct groups: (1) the fatty vehicle control group, which received the cell type specific standard media described above plus the vehicle dimethylsulfoxide (DMSO) < 0.1% v/v (D2438; Sigma-Aldrich) used for drugs dilution, without any drug or fatty acid supplement; (2) the fatty standard control group, which received only the standard culture media; (3) the defatting treatment group, which had the media supplemented with the combination of defatting drugs (0.01 mM glucagon mimetic and cAMP activator forskolin [F6886; Sigma-Aldrich], 0.001 mM PPAR  $\alpha$  ligand GW7647 [G6793; Sigma-Aldrich], 0.01 mM PXR ligand hypericin [56690; Sigma-Aldrich], 0.01 mM CAR ligand scoparone [254886; Sigma-Aldrich], 0.001 mM PPAR  $\delta$  ligand GW501516 [SML1491; Sigma-Aldrich], 0.4 ng/mL adipokine visfatin [SRP4908; Sigma-Aldrich] and 0.8 mM L-carnitine [C0283; Sigma-Aldrich]); and, (4) lean cells that were kept on standard media throughout. The defatting mixture of drugs was tested previously in rat hepatocytes and HepG2 cells (5, 6, 15). All groups had the media changed and sampled after 24 hours and 48 hours of treatment and the cells harvested for intracellular lipids quantification.

### **6.3.6 Isolation and culture of primary cholangiocytes and HIEC**

HIEC and cholangiocytes were isolated from human liver tissue using Collagenase Type 1A (C9891; Sigma-Aldrich) digestion for 1 hour at 37 °C. The resulting cell suspension was then sieved through a fine mesh, separated on a 33%/77% Percoll density gradient and cells retrieved from the interphase. This interphase mixed population of cells were then diluted in PBS, centrifuged and further immuno-magnetically separated with Dynabeads conjugated with cell-specific monoclonal antibody (anti-cluster of differentiation 31 [CD31] to purify HIEC [M0823, monoclonal mouse antibody anti-CD31, clone JC70A; Dako, Denmark] or anti-epithelial cell adhesion molecule [130-080-301, monoclonal mouse antibody, CD326, EpCAM-

FITC; Miltenyi Biotec, Bergisch, Germany] to purify cholangiocytes). The extracted cholangiocytes and HIEC were then plated on 96-well plates previously coated with rat tail collagen type 1; Cholangiocytes in DMEM 10% FCS supplemented with 5% GPS and the HIEC in Human Endothelial-Serum Free Media (CN:11111044; Gibco) supplemented with 10% heat-inactivated human serum (CR100; TCS Biologicals, Buckingham, UK) with 5% GPS. After an interval of 12 hours the media was changed to our standard specific culture medium. For cholangiocytes it was constituted of DMEM/ HAMS F-12 nutrient mixture (CN:21331-020; Gibco) 1:1 v/v, 5% GPS, hydrocortisone (0.4 µg/mL), cholera toxin (10 ng/mL) (C8052; Sigma-Aldrich), triiodothyronine (T3) ( $2 \times 10^{-9}$  mol/L) (T6397; Sigma-Aldrich), insulin (5 µg/mL), hepatocyte growth factor (10 ng/mL) (CN:100-39; Peprotech, Rocky Hill, NJ, USA) and epidermal growth factor (10 ng/mL) (CN:100-61; Peprotech). For HIEC, Human Endothelial-Serum Free Media supplemented with 10% heat-inactivated human serum, vascular endothelial growth factor (10 ng/mL) (CN:100-20C; Peprotech) and hepatocyte growth factor (10 ng/mL) was used. After isolation the cells were kept in an incubator at 37 °C in an atmosphere of 95% air/ 5% CO<sub>2</sub>.

Cholangiocytes and HIEC were cultured for 48 hours to reach confluence. After this period the culture media was changed, and the experimental groups assigned to the various cells. The intervention group received the standard culture medium supplemented with the defatting drugs (0.01 mM forskolin, 0.001 mM GW7647, 0.01 mM hypericin, 0.01 mM scoparone, 0.001 mM GW501516, 0.4 ng/mL visfatin and 0.8 mM L-carnitine). The control group was split into two, one that received vehicle (DMSO < 0.1% v/v) and a second one that received only the standard culture medium.

### **6.3.7 Cell viability assessment**

Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (M5655; Sigma-Aldrich). MTT is initially a yellow-coloured solution which turns purple formazan after reduction by reductase enzymes found within mitochondria of viable cells. DMSO is added to dissolve the purple formazan into a coloured solution. The readout was the difference between the values of the absorbance readings at 570 and 690 nm on a plate reader. The amount of purple formazan produced by control cells allows comparisons between the effects of different treatments. MTT assay was used to assess the cytotoxicity of the mixture of drugs and of the media supplemented with FAs.

#### **6.3.8 Oil red O staining**

Oil Red O staining was employed for quantifying LDs in the cytoplasm of the cells (O0625; Sigma-Aldrich). Following experimentation cells were fixed with buffered formaldehyde and the staining was carried out as previously described (16). Mayer's haematoxylin was used for the nuclear counter stain.

For quantification of staining in PHH four high power field (HPF) images from each experiment were selected. Positive areas of staining were calculated by a system of colour differentiation and the result expressed in percentage of the total area of the image using ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA.

#### **6.3.9 Intracellular triglyceride quantification**

At the end of the incubation period, cells were washed with PBS and harvested with gentle scraping. Intracellular lipids were retrieved using the detergent TERGITOL™ Type NP-40 (NP40S; Sigma-Aldrich) followed by lipase incubation to break down triglycerides into FAs and glycerol. Intracellular triglyceride was measured using a colorimetric assay (ab65336; Abcam, Cambridge, MA, USA) based on a reaction of glycerol oxidation producing colour.

The concentration of triglycerides was normalised to control and expressed as nmol per million of cells.

#### **6.3.10 Ketone bodies measurement**

Ketone bodies were quantified in cell supernatants using a commercially available kit (MAK134; Sigma-Aldrich) as per the manufacturer's instructions and detect 3-hydroxybutyric acid (BOH) and acetoacetic acid (AcAc).

#### **6.3.11 Statistical analysis**

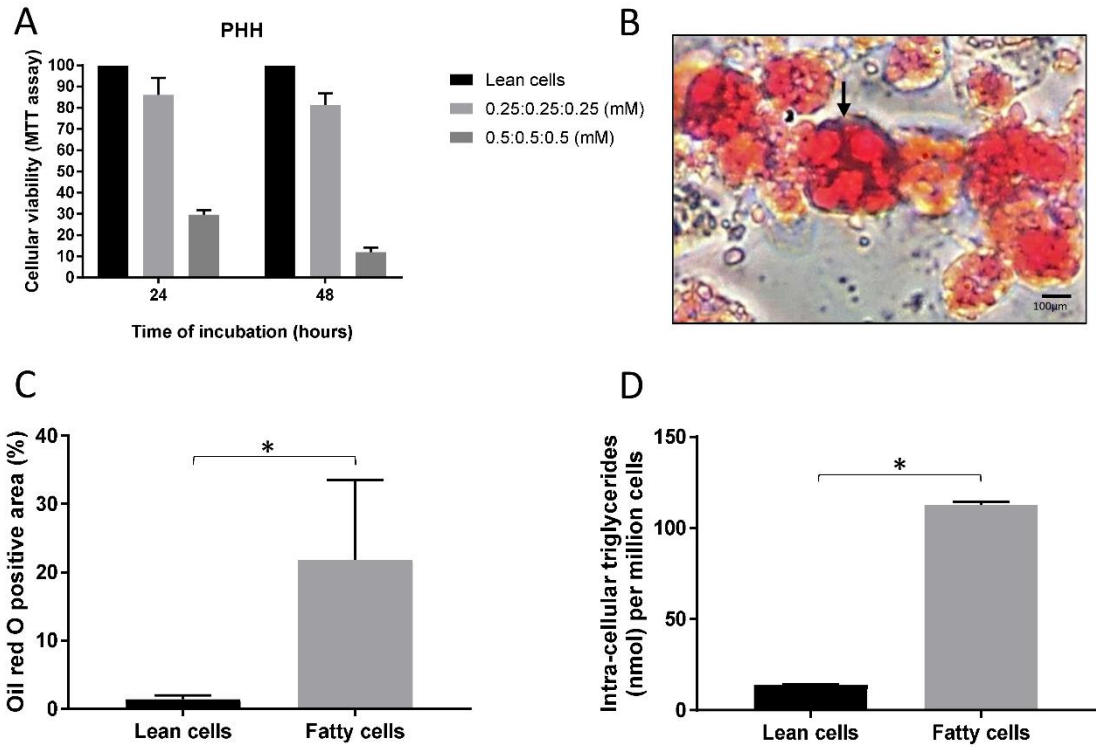
Continuous variables were expressed as median/ interquartile range or range and categorical variables as absolute number/ frequency (%). Comparison between groups was performed using Mann–Whitney *U* test or two tailed t-test for continuous variables. The statistical level of significance was  $p < 0.05$ . GraphPad Prism version 6.04 for Windows, GraphPad Software, La Jolla California USA was used for statistical analysis and graph creation.

### **6.4 Results**

#### **6.4.1 Induction of steatosis in PHH**

Incubation of PHH for 48 hours with media supplemented with the combination of FAs increased the median intracellular triglyceride concentration approximately 8-fold, from 14.01 (range 13.69–14.02) to 112.64 (111.65–113.30) nmol/million of cells ( $p < 0.001$ ). The PHH oil red O staining area increased significantly from 1.4% (0.9–1.7) to 21.8% (14.7–32.0) (approximately 14-fold) ( $p < 0.05$ ). The cellular viability of PHH after 48 hours of incubation with the media supplemented with FAs using the MTT-assay was 81% (range 76–87%) in comparison with lean cells kept in the standard media throughout. The results from the steatosis induction period are in Figure 6.2.

**Figure 6.2: Results of fat loading of primary human hepatocytes.**



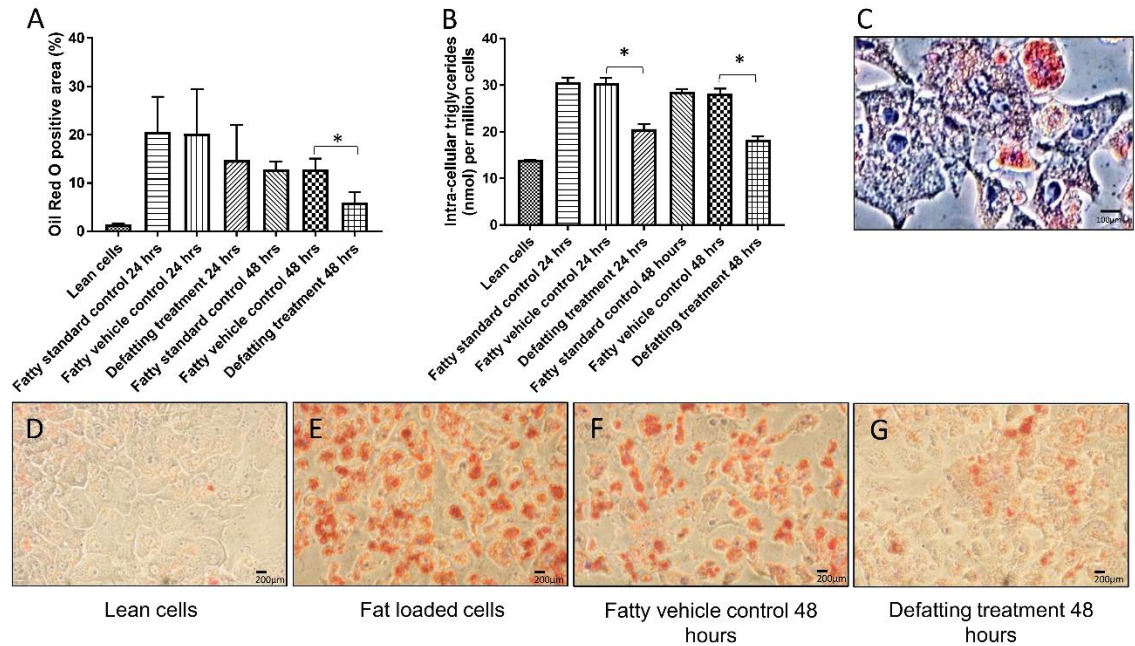
Panel A: The supplementation of the media with the combination of fatty acids resulted in a cell viability rate of 81% after 48 hours of incubation. Panel B: Oil red O staining image of primary human hepatocytes (PHH) at the end of the fat loading period. There is predominance of large lipid droplets displacing the nucleus of the cells to the periphery (black arrow). Panel C: At the end of 48 hours of fatting load there was a significant increase of 14-fold of the positive area of oil red O. Panel D: Intracellular triglycerides increased 8-fold within 48 hours of incubation with fatty acids. Data report the median of three separate experiments performed in quadruplicate and errors bars the interquartile range. Comparisons performed using two-tailed t-test.  $*= p < 0.05$ .

## 6.4.2 Defatting of PHH

### 6.4.2.1 Oil red O analysis

Digital analysis of oil red O staining of cytoplasmic LDs demonstrated that the combination of drugs lead to a decrease from 28% [20.27% (14.67–32.03%) to 14.77% (11.70–22.05%),  $p = 0.315$ ] in median positive area of oil red O staining within 24 hours of treatment and 54% within 48 hours [12.85% (11.07–15.80%) to 5.99% (4.24–8.61%),  $p = 0.002$ ] compared to the fatty vehicle control hepatocytes alone (Figure 6.3). The positive area of oil red O was comparable between the fatty vehicle control cells and the fatty standard control cells after 24 hours [20.27% (14.67–32.03%) vs. 19.98% (15.03–31.70%),  $p > 0.999$ ] and 48 hours of treatment (12.85% (11.07–15.80%) vs. 13.04% (12.01–16.02%),  $p > 0.999$ ). The same pattern was seen for the other parameters analysed, therefore, comparisons with the defatting group were made using the fatty vehicle control group. In addition to the decrease in the total area of cytoplasmic LDs (expressed as percentage oil red O positive area) in the defatting group, it morphologically appeared to switch from a macrovesicular to a microvesicular appearance (Figure 6.3).

**Figure 6.3: Defatting of fat loaded primary human hepatocytes (PHH).**



Panel A: The positive area of oil red O of the defatting treatment group was reduced by 28% in comparison with the vehicle control group over 24 hours and 54% over 48 hours. Panel B: Intracellular triglyceride levels of the defatting treatment group were reduced by 32% within 24 hours of treatment and 35% within 48 hours, in comparison with the fatty vehicle control group. Panel C: Oil red O staining picture of primary human hepatocytes (PHH) of the defatting group at the end of the 48 hours of treatment. There is a predominance of small lipid droplets in the cytoplasm of the cells and the nucleus is in its usual position. Series 2: shows a series of oil red O staining pictures from PHH in culture at different time points of the experiments. Panel D shows lean cells in culture, after the incubation with fatty acids they become loaded with fat (Panel E). Those fat loaded PHH were then incubated with only the vehicle of the drugs for 48 hours and the lipid content decreased over time (Panel F) or had the defatting treatment that showed the significant higher decrease in the area of oil red O (Panel G). Data report the median of three separate experiments performed in quadruplicate and errors bars the interquartile range. Comparisons performed using two-tailed t-test.  $*=p < 0.05$ .

#### **6.4.2.2 Intracellular triglyceride quantification**

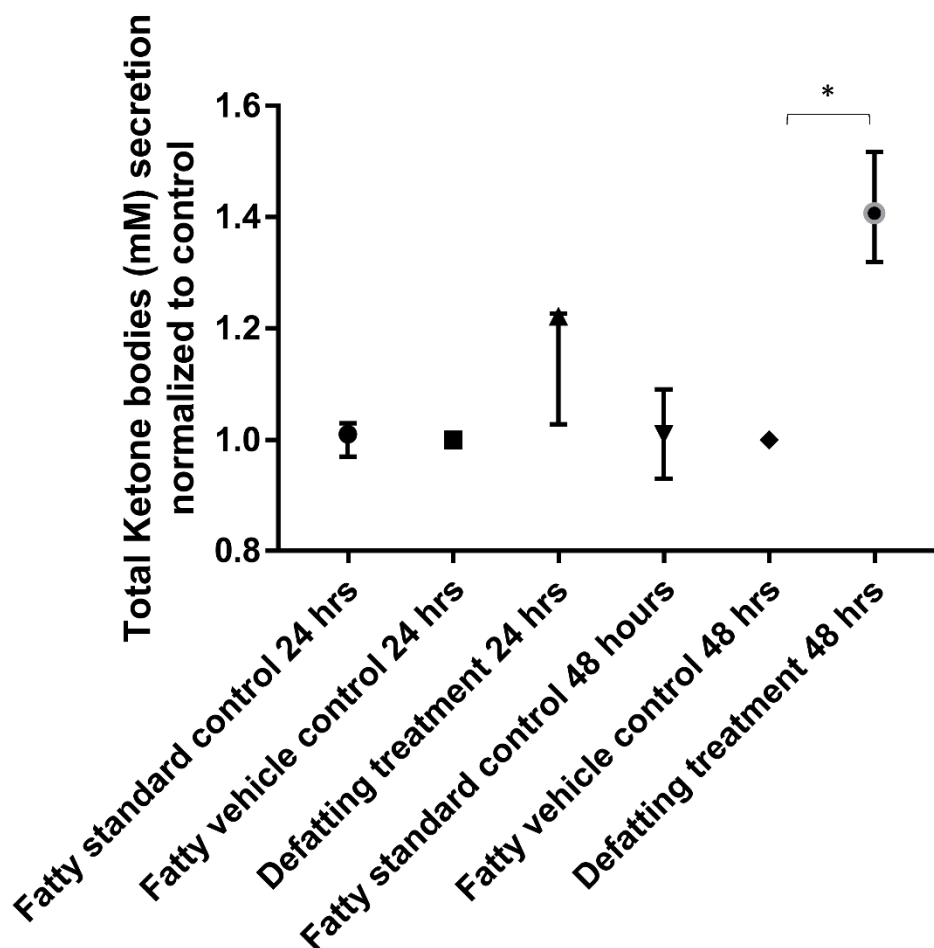
Treatment with the defatting cocktail decreased the median concentration of intracellular triglycerides by 32%, from 30.51 nmol/million of cells (range 30.18–31.50) in the fatty vehicle control group to 20.61 nmol/million of cells (range 20.28–21.60) in the defatting group within 24 hours,  $p = 0.012$ . After 48 hours, it reduced 35%, from 28.24 nmol/million of cells (range 26.88–29.24) to 18.30 nmol/million of cells (range 18.30–18.96) ( $p < 0.001$ ). Intracellular triglyceride concentration reduced over time once the cells were removed from the fattening media by approximately 5-fold within 24 hours (Figure 6.3). There was no difference between intracellular triglycerides levels of the fatty control vehicle alone and the fatty standard control group ( $p > 0.999$ ).

#### **6.4.2.3 Fatty acids $\beta$ -oxidation induction**

The defatting cocktail induced a median increase in the release of total ketone bodies in the supernatant of PHH of 1.22-fold (range 1.02–1.26) ( $p = 0.070$ ) after 24 hours of treatment and 1.40-fold within 48 hours (range 1.31–1.52) ( $p = 0.002$ ) (Figure 6.4) when compared to the fatty vehicle control group alone at each respective time point. The release of total ketone bodies in the supernatant was similar between both control groups.



Figure 6.4: Release of total ketones in the supernatants.



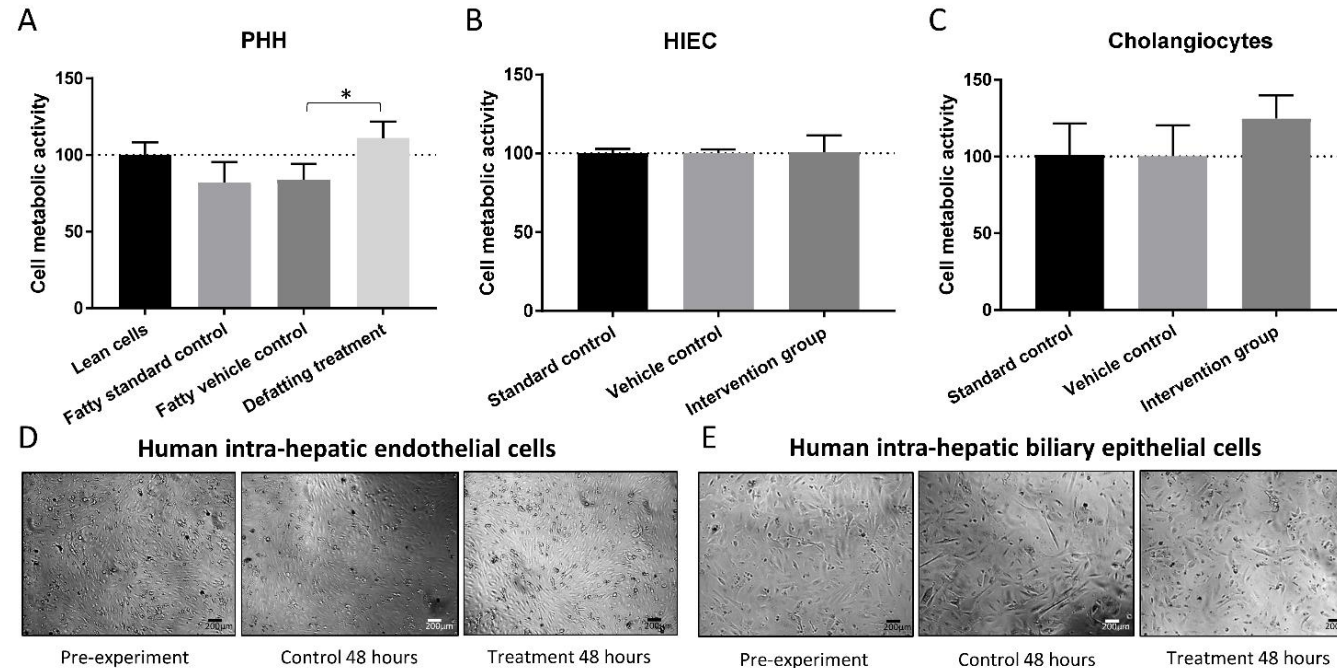
Fat loaded primary human hepatocytes that had the defatting treatment showed an increase in cell culture supernatant levels of total ketone bodies of 1.22-fold over 24 hours and 1.40-fold over 48 hours. Data reports the median of three separate experiments performed in quadruplicate and errors bars the interquartile range. Comparisons performed using two-tailed t-test.  $\ast = p < 0.05$ .

### **6.4.3 Impact of the defatting cocktail on cell viability**

#### **6.4.3.1 Effects on PHH**

After 48 hours of treatment with the defatting cocktail MTT assay showed an increase in the viability of PHH of 11% (6–15%) in comparison with the fatty vehicle control cells ( $p = 0.048$ ). Cellular viability was similar between the control groups. Phase contrast light microscopy suggested that the defatted hepatocytes were more adherent and spread on the wells in comparison with the fatty vehicle control cells which continued to die (Figure 6.5).

**Figure 6.5: Assessment of the cytotoxicity of the defatting cocktail to human cells of the liver via MTT assay.**



Panel A: The toxicity of the defatting cocktail was tested in primary human hepatocytes (PHH) and results showed a significant improvement of 11% in viability of the defatting treatment group compared with the fatty vehicle control group. Panel B: Treatment of human intrahepatic endothelial cells (HIEC) with the drugs had no effect on cell viability compared with the control groups. Panel C: treatment of cholangiocytes with the defatting cocktail did not demonstrate any cytotoxic effect to the cells and indicated a slight improvement in viability compared to the control groups. Panels D and E: Phase contrast microscopy showing representative images of HIEC (Panel D) and cholangiocytes (Panel E) at different time points. No gross modifications in cell integrity were observed in either cell type which was consistent and supportive of the MTT data. Data report the median of three separate experiments performed in quadruplicate and errors bars the interquartile range. Comparisons performed using two-tailed t-test.  $\ast = p < 0.05$ .

#### 6.4.3.2. Effects of the defatting cocktail on other liver cell types

Incubation of cholangiocytes and HIEC with the defatting cocktail was not cytotoxic. There was no difference in viability between HIEC in the treated group and the vehicle control group after 48 hours of incubation (100% [97–101] vs. 100% [90–113],  $p > 0.999$ ). For cholangiocytes the supplementation of the media with those drugs improved cellular viability in 25% although the difference did not reach statistical significance (125% [75–166] vs. 100% [57–116],  $p = 0.413$ ). Detailed data is presented in Figure 6.5.

### 6.5 Discussion

Defatting of steatotic rat livers using a cocktail of drugs in a model of *ex situ* normothermic machine perfusion was shown to be feasible and a potentially promising translational approach to improve the utilisation of steatotic donor livers for transplantation (2). However before considering the translational application of such interventions to whole human donor livers, the efficacy and cytotoxicity of these agents to human liver cells needed to be evaluated since there is inherent variability in responses to drugs between species and different cell lines (7–9). Our study demonstrates for the first time using PHH that the defatting cocktail was able to reduce its lipid content *in vitro* enhancing beta-oxidation of FAs. Moreover, we have shown that the drugs were not toxic to PHH, HIEC or cholangiocytes. The latter being crucial in the clinical setting where these cells are targets in ischaemia-reperfusion injury and ischaemic cholangiopathy (17, 18).

Steatosis is a frequent reason for livers being deemed non-transplantable (19). This is because these organs are more susceptible to ischaemic injury during cold preservation and thereafter are at a high risk of graft dysfunction after transplantation (1–4, 20, 21). Current strategies for transplantation of steatotic deceased donor livers rely mainly on the prevention of additional

risk factors, such as limiting cold ischaemia times, using low risk donors and selecting low risk recipients (22). An intervention which may decrease post-transplant complications for such livers has been described in the context of living donation. Living donors that had their diet supplemented with  $\Omega$ -3 FAs one month before organ procurement appears to be associated with fewer postoperative complications. Although this approach shows promise, it is not applicable in the context of deceased donor organs (22, 23).

Nagrath *et al.* 2009 tested a cocktail of drugs in an *in vitro* model of steatotic rat hepatocytes and showed a decrease in the intracellular triglyceride levels of 31% within 48 hours (5). This cocktail was tested then by Yarmush *et al.* 2016 in an *in vitro* model of hepatoblastoma cells (HepG2) loaded with lipids via FAs supplementation. The combination of drugs promoted a reduction of 83% in intracellular triglycerides within 48 hours of treatment under hyperoxic conditions (6). We have employed the same cocktail of drugs in our experiments and in PHH we found a reduction of 35% over the same time period. This reduction was more significant considering the positive area occupied by LDs (54%). Our data suggests that this finding is likely to be related to the reported decrease in size of the LDs gaining appearance of microvesicular steatosis. This decrease in macrovesicular steatosis was associated with a continuous increase in the production of ketone bodies with the defatting cocktail in comparison with fatty control cells further than 24 hours, a product of incomplete oxidation of FAs in the mitochondria.

The defatting cocktail consists of: nuclear ligands for peroxisome proliferator-activated receptors (PPAR  $\alpha$ - ligand GW7647; and, PPAR  $\delta$ - ligand GW501516) to stimulate the transcription of lipid oxidation/exportation factors (24); an insulin-mimetic adipokine visfatin associated with lowering triglyceride levels in the liver (25); forskolin, a glucagon mimetic molecule known to stimulate cyclic AMP-driven  $\beta$ -oxidation of lipids and ketogenesis (26);

pregnane X Receptor (PXR) ligand hypericin that is reported to improve the transcription of the cytochrome P450 (CYP) 3A4 monooxygenase which can increase metabolism of a range of drugs in hepatocytes (27); constitutive androstane receptor (CAR) ligand scoparone (6,7-dimethoxycoumarin) that acts to promote transcription of beta-oxidation enzymes, such as carnitine palmitoyltransferase 1 (28), and finally a supplement of L-carnitine, fundamental in the transport of FAs across the inner mitochondrial membrane (29).

In brief, forskolin activating glucagon membrane receptors can stimulate the adenosine monophosphate (cAMP) – protein kinase A pathway that regulates the trafficking of cytoplasmic lipases to the surface of LDs (30, 31). The glycerol and FAs released from the breakdown of LDs could potentially not only serve as substrates for the cell metabolism but also as ligands to nuclear receptor (peroxisome proliferator receptor [PPAR] and liver X receptors [LXR]) increasing the transcription of enzymes involved in the catabolism of FAs in the mitochondria and peroxisome (32, 33). The other drugs (GW7647, GW501516, hypericin, scorparone) also act as ligands to other nuclear receptors (pregnane X receptors and androstane receptors) boosting the transcription of key enzymes in lipid metabolism (34, 35). Cytosolic fatty acid reacts with ATP generating fatty acyl-CoA. Acyl-CoA in turn reacts with apolipoprotein B to generate lipoproteins to be exported from the cell and/or reacts with the hydroxyl group of carnitine via carnitine palmitoyltransferase 1. Acyl-carnitine is transported inside the mitochondria by a Carnitine-acyl-CoA transferase and a carnitine is transferred outside. Acyl-CoA is processed by  $\beta$ -oxidation then allowing ketogenesis or complete oxidation via the Krebs cycle and the electron transport chain with the production of adenosine triphosphate (ATP) (36). Therefore, acting through different pathways, this combination of drugs accelerates the process of intracellular triglycerides exportation and improves mitochondrial oxidation of FAs (5, 37). Hence the defatting cocktail not only reduces

intracellular LD content, but it also serves to enhance lipid metabolism and potentially increase intracellular ATP. This increased ATP content can potentially improve the poor outcome of steatotic livers during either machine perfusion or after clinical transplantation as loss of energy reserves is an important reason for graft dysfunction (38).

As already stated, experiments testing the defatting cocktail with rat hepatocytes and HepG2 cells do not wholly reflect the responses of PHH. For example, the expression of enzymes involved in drug metabolism as cytochrome P450 is variable between these cells and PHH (7, 10). Consequently, such experiments although informative, may underestimate the real cytotoxicity or metabolic effect of therapeutic interventions in human livers (7, 8, 10, 39). Therefore, PHH remain the choice of cells for the study of cytotoxicity and the resultant metabolic effect of drugs in human livers (11, 12, 39).

The present study to the best of our knowledge is the first to examine the cytotoxicity and metabolic effect of a combination of drugs intending to promote defatting of fat loaded PHH in an *in vitro* model. It was shown that the defatting cocktail effectively decreased the lipid content of PHH *in vitro*, improving cellular viability and mitochondrial oxidation of FAs. No less importantly, we have tested the cytotoxicity of the drugs to HIEC and cholangiocytes. The information that the drugs are not toxic to these cells is an important and reassuring step before moving towards translational experiments for the delivery of the defatting cocktail to whole human donor livers during extra-corporeal normothermic machine perfusion.

Despite the experimental evidence discussed, defatting of human livers remains challenging and under researched. One limitation of our study is that we have not explored what could be the impact of flow and the effect of ischaemia-reperfusion on the defatting process of PHH. It was suggested recently in the literature by Yarmush *et al.* 2017 that cultured HepG2 cells can

have the time for defatting shortened from 48 hours to 4–6 hours when submitted to conditions of flow using this cocktail of drugs (37). In addition, machine perfusion and/or clinical transplantation of livers will involve a period of ischaemia and this may have an effect upon defatting (38).

## **6.6 Conclusion**

Using an *in vitro* model of PHH, our study demonstrates for the first time that pharmacological interventions can be used to lower intracellular triglycerides stores and promote higher rate of FAs mitochondrial  $\beta$ -oxidation. In addition, the drugs were shown to be not toxic to PHH, HIEC and cholangiocytes. Hence, the present study supports future translational experiments involving the described defatting cocktail in steatotic human livers.



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## Chapter 7

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# MANIPULATION OF LIPID METABOLISM DURING NORMOTHERMIC MACHINE PERFUSION: EFFECT OF DEFATTING THERAPIES ON DONOR LIVER FUNCTIONAL RECOVERY

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**A version of this manuscript has been accepted for publication**

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### *Liv Transplant*

Y.L.B. designed the experiment, performed the experiment, interpreted the data and wrote the manuscript. J.A. assisted in the experiments. A.P.C.S.B. assisted in some of the experiments. L.W. assisted with the technicalities of the laboratory tests. G.R. and S.H. performed the histopathological assessment of the samples. D.F.M. and H.M. offered surgical support for the experiments. R.H.B. assisted in editing the manuscript. S.C.A. designed the experiments, assisted in the interpretation of the data and editing the manuscript.

## 7.1 Abstract

**Background:** Strategies to increase steatotic donor livers' utilisation are required to tackle the mortality on the transplant waiting list. We aimed to test the efficacy of pharmacological enhancement of the lipid metabolism of human livers during *ex situ* normothermic machine perfusion to promote defatting and improve the functional recovery of the organs.

**Methods:** Ten livers discarded because of steatosis were allocated to: a defatting group that had blood-based perfusate supplemented with a combination of drugs to enhance lipid metabolism or a control group that received perfusion fluid with vehicle only. Steatosis was assessed using tissue homogenate and histological analysis. Markers for lipid oxidation and solubilisation, oxidative injury, inflammation and biliary function were evaluated by ELISA, immunohistochemistry and in-gel protein detection.

**Results:** Treatment reduced tissue triglycerides by 38% and macrovesicular steatosis by 40% over 6 hours. This effect was driven by increased solubilisation of triglycerides ( $p = 0.04$ ); and, mitochondrial oxidation as assessed by increased ketogenesis ( $p = 0.008$ ) and adenosine triphosphate synthesis ( $p = 0.01$ ) associated with raised levels of the enzymes ACOX-1, CPT1A and acetyl-CoA synthetase. Concomitantly defatted livers demonstrated enhanced metabolic functional parameters such as urea production ( $p = 0.03$ ), higher vascular flows ( $p = 0.03$ ), lower release of alanine aminotransferase ( $p = 0.049$ ) and higher bile production ( $p = 0.008$ ) with a higher bile pH ( $p = 0.03$ ). The treatment downregulated the expression of markers for oxidative injury, activation of immune cells (CD-14; CD-11b) and reduced the release of inflammatory cytokines in the perfusate (TNF- $\alpha$ ; IL-1 $\beta$ ).

**Conclusion:** Pharmacological enhancement of intracellular lipid metabolism during normothermic machine perfusion led to defatting of steatotic human livers within 6 hours. It

also improved the intracellular metabolic support to the organs leading to successful functional recovery and attenuated expression of markers of reperfusion injury.

## **7.2 Introduction**

Steatosis is caused by the abnormal metabolism of fatty acids in hepatocytes, resulting in intracytoplasmic accumulation of triacylglycerol as lipid droplets (LD) (1). In the context of organ donation, livers presenting with large intracytoplasmic LD displacing the cell nucleus (macrovesicular steatosis) are more vulnerable to ischaemic-reperfusion injury (IRI) during standard static cold storage (SCS). This is mainly due to impaired mitochondrial function, poor microcirculation and exaggerated inflammatory response leading to tissue damage (2). The exacerbated IRI is then associated with impaired early functional recovery and a high risk of early allograft dysfunction (2–4). Therefore, steatosis or fatty livers is one of the main reasons for declining donor livers worldwide (2, 5).

Animal models have shown that normothermic machine perfusion (NMP) alone is able to improve intracellular lipid metabolism and promote steatosis reversal or defatting (6, 7). However, a recent study involving NMP of steatotic human livers for 24 hours did not show a decrease in tissue steatosis (5). In a murine model, a combination of drugs (peroxisome proliferator activated receptor [PPAR] $\alpha$  ligand GW7647, PPAR $\delta$  ligand GW501516, pregnane X receptor [PXR] ligand hypericin, the constitutive androstane receptor [CAR] ligand scorparone, the cyclic adenosine monophosphate [cAMP] activator forskolin, and the insulin-mimetic adipokine visfatin), administered during NMP, was able to enhance lipid metabolism and reduce tissue triglycerides (TG) by 50% over 3 hours of perfusion (6). Similar results were induced by glial cell line–derived neurotrophic factor (GDNF) (8). However, whether those

observations could be reproduced in human livers is still to be determined. In addition, those organs had not been exposed to ischaemic injury, as is the case in liver transplantation; therefore, whether it would affect the defatting process remains under-researched.

We aimed to study the feasibility of the delivery of this combination of drugs to steatotic human donor livers undergoing end-ischaemic NMP within the regular process of organ donation and cold preservation. The impact of this intervention on the mobilization and metabolism of intracellular lipids leading to defatting was assessed, together with its effects on recovery of the metabolic activity of the organs.

### **7.3 Materials and Methods**

#### **7.3.1 Study design and source of discarded donor livers**

Ten human livers discarded for transplantation due to surgical macroscopic assessment of steatosis were submitted to NMP for 12 hours after variable periods of SCS. They were randomly allocated to the experimental groups using the covariate adaptive randomization method that accounted for donor type and cold ischaemia time (CIT). The defatting group had the perfusion fluid supplemented with a combination of drugs to improve lipid metabolism (Defatting cocktail) (5 livers) and a control group (5 livers) which received vehicle only in the perfusion fluid (dimethyl-sulfoxide [DMSO] < 0.01%). The comprehensive protocols for sampling (including core needle liver biopsies, perfusate and bile analysis) and data collection are presented in the supplementary material.

All study livers were originally retrieved with the intention of transplantation as per policy of the National Health Service Blood and Transplant (NHSBT). The authorisation for research use of the organ was obtained by the specialist nurse in organ donation in accordance with NHSBT guidelines. Ethical approval for the study was obtained from the London-Surrey Borders

National Research Ethics Service and the NHSBT Ethics Committee (references 13/LO/1928 and 06/Q702/61, respectively).

### **7.3.2 Liver perfusion procedure**

After arrival at our centre, the organs were prepared following the standard bench preparation as described elsewhere (9). The cystic duct was ligated, and a 12 French bile cannula inserted in the common bile duct. The liver was flushed via portal vein (PV) and hepatic artery (HA) with two litres of 5% glucose, transferred to the reservoir of the disposable circuit and then perfusion was started.

Liver Assist (Organ Assist, Groningen, Netherlands) was the device used for the perfusions. Temperature was set initially at 20 °C increasing gradually to 37 °C within the first 30 minutes. Initial pressure on the HA was 30 mmHg and 5 mmHg on the PV. Both were raised steadily with incremental increases in temperature reaching 60 mmHg and 10 mmHg respectively at 30 minutes. Oxygen supply was adjusted to achieve partial pressure of 20-30 kPa in the perfusate on the arterial circuit. The perfusate consisted of 3 units of packed red blood cells, 5% w/v human albumin solution and additional drugs as specified in the supplementary Table S7.1.

### **7.3.3 The defatting cocktail of drugs**

A previously published cocktail of drugs (6) (10 µM of forskolin; 1 µM of GW7647; 10 µM of hypericin; 10 µM of scoparone; 0.4 ng/mL of visfatin; 1 µM of GW501516) was supplemented with L-carnitine 0.8 mM diluted in DMSO and added in the perfusate for the defatting group when the perfusate reached 37 °C. The control group received an equal amount of DMSO (<0.01% v/v) in the perfusate within the same timeframe. Drugs were obtained from Sigma-Aldrich, St. Louis, MO, USA and a list is provided in the supplementary section.



#### **7.3.4 Histological evaluation**

Tissue biopsies were embedded in paraffin and cut in sections of 4  $\mu\text{m}$ . Staining with haematoxylin and eosin (H&E) was performed to assess necrosis, grade of steatosis, pre-existing acute or chronic liver injury and periodic-acid Schiff (PAS) for glycogen stores. Macrovesicular steatosis (MaS) was defined as the presence of a large LD filling up the hepatocytes and displacing the nucleus to the periphery. Microvesicular steatosis (MiS) refers to the presence of numerous small LDs in the hepatocyte ('foamy' aspect) without affecting cell nuclei position. MaS was graded based on the percentage of hepatocytes involved (None: < 5%; Mild: 5–30%; Moderate: 30–60%; Severe: > 60%). MiS was reported as present or absent. Histological assessment was done under lower magnification (4–10 $\times$ ) and confirmed under higher magnification (20–40 $\times$ ), if required. It was conducted by two independent pathologists (GR and SR) blinded to the study group.

#### **7.3.5 Assessment of lipid metabolism**

Perfusate total cholesterol (t-cholesterol), high density lipoprotein (HDL) and TG (P-TG) were measured at the hospital clinical laboratories. Tissue TG (T-TG) was assessed from tissue homogenates using a commercially available kit (10010303; Cayman chemical, Miami, Florida, USA) following manufacture's guidelines. Total ketone bodies (3-hydroxybutyric acid and acetoacetic acid) were measured in the perfusate using a commercial kit (MAK134; Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions.

In-gel fluorescent protein staining was performed to investigate changes across groups in transcription of three key enzymes in the intracellular lipid metabolism: (1) Acetyl-CoA synthetase promotes the reaction of coenzyme A (CoA) with FAs generating the substrate required for mitochondrial  $\beta$ -oxidation of FAs; (2) The liver specific 1A form of carnitine

palmitoyltransferase (CPT1A) responsible for the shuttle of acetyl-CoA into the mitochondrial matrix for oxidation; and (3) Peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), an essential enzyme for the peroxisomal oxidation of medium to long chain FAs. Detailed techniques and the list of antibodies are provided in the supplementary material.

### **7.3.6 Assessment of the liver metabolism**

Parameters used in our unit as indicators of ongoing liver metabolism were applied to define whether organs would be potentially transplantable (10). Other parameters, such as the dynamics of lactate clearance, bile quality (assessed by bile pH), urea production, release of transaminases in the perfusate and oxygen uptake were also investigated. For analysis of dynamic changes in lactate metabolism over time the area under the curve (AUC) of lactate concentrations in the perfusate between 0 and 12 hours was analysed. Oxygen uptake was calculated as the difference between the oxygen inflow and the outflow in kPa.

### **7.3.7 Biochemistry analysis**

Markers of hepatocellular injury (alanine aminotransferase [ALT], aspartate aminotransferase [AST]), biliary injury/function (gamma-glutamyl transpeptidase [GGT]) and protein release (albumin and total protein) were measured in the perfusate (time points in the sampling protocol).

### **7.3.8 Cellular energy status assessment**

Levels of adenosine triphosphate (ATP) were assessed using a fluorometric commercial kit from liver tissue homogenates (MAK190; Sigma-Aldrich). Levels were normalised to mg of protein.

### **7.3.9 Assessment of oxidative injury and inflammation**

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections for the expression of the marker of cell membrane phospholipid peroxidation 4-hydroxynonenal (4-HNE). To assess tissue inflammation, the cluster of differentiation (CD)-11b, an integrin on the surface of activated leukocytes; and, the CD-14, a lipopolysaccharide receptor which is part of the toll-like receptor 4 signalosome and participates in the development of the IRI, were investigated. For the detailed technique and antibodies list refer to supplementary material. Staining expression was scored using a semiquantitative scoring system, the modified immunoreactive score (IRS) (11). This takes into consideration intensity of staining (0: no colour reaction; 1: mild reaction; 2: moderate reaction; 3: intense reaction) and its distribution (0: no positive cells; 1: < 10% positive cells; 2: 10–50% positive cells; 3: 51–80% positive cells; 4: > 80% positive cells). A final score between 0 and 12 is obtained by multiplying the two parameters.

Perfusate TNF- $\alpha$ , interleukin (IL)-10 and IL-1 $\beta$  levels were determined using sandwich enzyme-linked immunosorbent assay (ELISA) (RAB0476, RAB0244, RAB0273; Sigma-Aldrich). 8-hydroxy-2-deoxyguanosine (8-OHdG) was assessed in the perfusate using a commercially available kit (ab201734; Abcam, Cambridge, MA, USA) following the manufacturer's instruction. All samples were tested in duplicate.

### **7.3.10 Statistical analysis**

Continuous variables were expressed as median with interquartile range (IQR) and categorical variables as an absolute number with percentage frequencies. Comparisons between groups were performed using two-tailed Fisher's exact test for categorical variables, Mann–Whitney *U* test or Student's *t* test for independent continuous variables and Wilcoxon signed-rank test for repeated measurements over time on the same sample. Correlation between variables were

analysed using Pearson's correlation coefficient. Area under the curve was calculated using the trapezoidal rule. The statistical level of significance was set at  $p < 0.05$ . GraphPad Prism (version 6.04 for Windows, GraphPad Software, La Jolla, USA) software was used for all statistical analyses and graph creation.

## **7.4 Results**

### **7.4.1 Donor demographics and perfusion parameters**

Overall median donor age was 51 years (IQR: 47–58), median donor risk index was 2.02 (1.99–2.09) and median CIT 737 minutes (717–805). Each study group included three donors after brain death (DBD) and two donors after circulatory death (DCD) livers. The groups were comparable in terms of donor characteristics and preservation times. Details provided in Table 7.1 and S7.2.

**Table 7.1: Donor demographics, liver characteristics and machine perfusion data.**

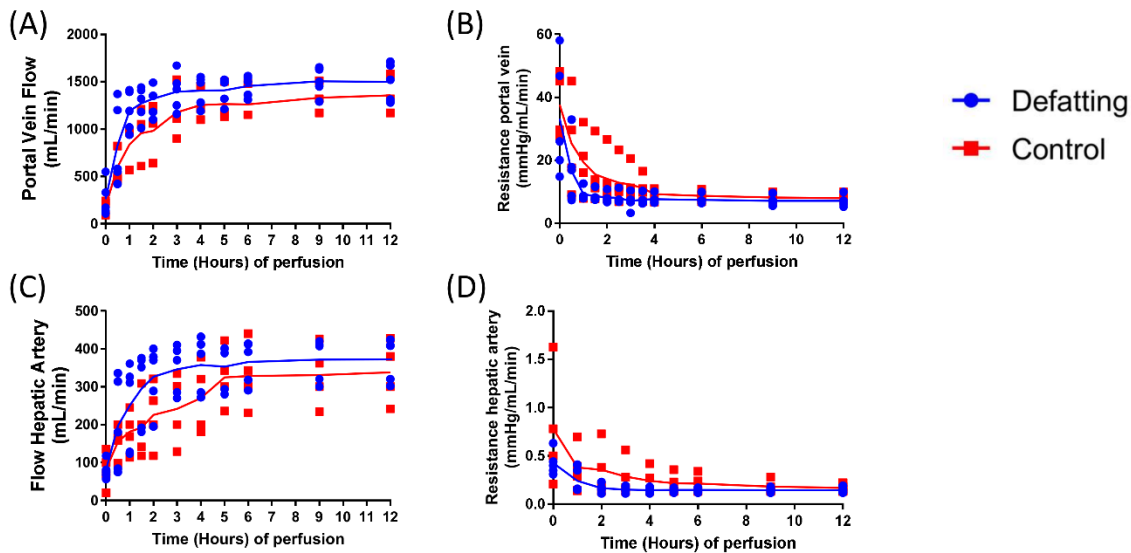
Parameter	Defatting ( <i>n</i> = 5)	Control ( <i>n</i> = 5)	<i>p</i> -value
<b>Donor information</b>			
Age, years	52 (47–61)	49 (44–66)	0.98
DCD livers	2 (40%)	2 (40%)	> 0.99
Sex, male	3 (60%)	3 (60%)	> 0.99
Height (cm)	174 (162–184)	172 (167–184)	0.72
Bodyweight (kg)	86 (75–100)	95 (75–105)	0.67
Body mass index (kg/m <sup>2</sup> )	30 (28–34)	28 (25–34)	0.52
Donor risk index	2.04 (1.91–2.80)	2.00 (1.83–2.09)	0.22
UK donor liver index	1.14 (0.98–1.77)	1.28 (0.94–1.76)	0.94
ET donor risk index	1.85 (1.75–3.11)	2.00 (1.98–2.50)	0.75
Peak ALT (IU/L)	66 (39–99)	104 (39–109.5)	0.38
Peak GGT (IU/L)	110 (46–239)	355 (31–369)	0.29
Days on ventilator	2 (2–4)	3 (1–4)	0.64
<b>Liver characteristics</b>			
Liver weight (grams)	1874 (1731–2362)	2130 (1775–2228)	0.93
Donor warm ischaemic time (minutes)	12 (10–14)	13 (13–13)	0.70
Cold ischaemia time (hours), DCD	12:54 (12:09–13:40)	12:50 (12:20–13:20)	0.65
Cold ischaemia time (hours), DBD	12:34 (12:07–13:08)	12:03 (10:21–12:09)	0.29
<b>Machine perfusion parameters</b>			
Lactate (mmol/L)			
Start (0-hour)	11.1 (9.2–14.4)	10.6 (9.4–11.8)	> 0.99
Highest	11.6 (10.4–16.3)	14.7 (12.1–18.8)	0.38
Lowest	0.9 (0.3–1.8)	1.6 (0.7–11.7)	0.14
Last (12–hours)	1.9 (0.6–2.7)	2.2 (1.0–16.0)	<b>0.003</b>
Sodium bicarbonate 8.4% supplementation (mL)	20 (15–27)	40 (30–90)	<b>0.04</b>
Total bile production (mL/hour)	1.7 (1.6–2.6)	0.6 (0.2–1.6)	<b>0.02</b>
Bile pH (12 hours)	7.84 (7.71–7.95)	7.34 (7.10–7.60)	<b>0.02</b>
Median arterial flow (mL/min)	360 (283–405)	286 (167–343)	0.09
Median portal vein flow (mL/min)	1316 (1232–1515)	1020 (900–1302)	0.056
Viability achievement	5 (100%)	2 (40%)	<b>0.04</b>

**Abbreviation:** DCD- Donation after circulatory death; UK- United Kingdom; ET- Eurotransplant; ALT- Alanine aminotransferase; GGT- Gamma-glutamyltransferase; DBD- Donation after brain death.

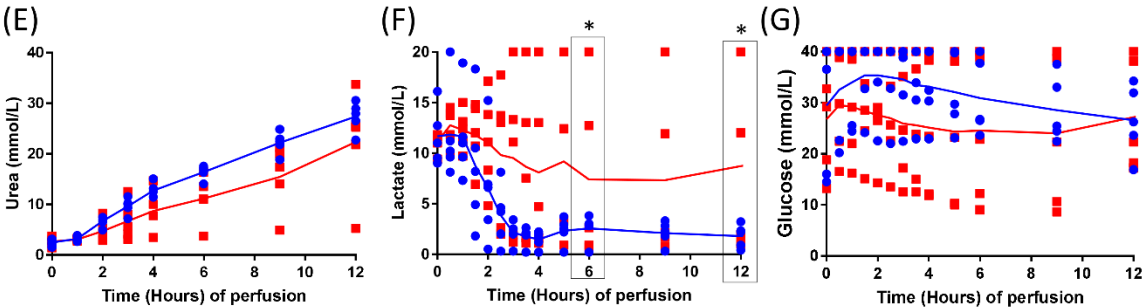
Treated livers developed higher median PV flows ( $p = 0.056$ ) with lower vascular resistance ( $p = 0.005$ ) from the start until 12 hours of perfusion. There was a trend of higher median HA flows in the defatting group ( $p = 0.09$ ) with a significantly lower HA resistance ( $p = 0.003$ ) during this period. There was a strong correlation between T-TG and flows at times 6 and 12 hours on the HA ( $r = -0.66$ ,  $p = 0.04$  and  $r = -0.68$ ,  $p = 0.03$ , respectively) and PV ( $r = -0.79$ ,  $p = 0.007$  and  $r = -0.81$ ,  $p = 0.004$ , respectively). Details are shown in Figure 7.1.

**Figure 7.1: Perfusion parameters.**

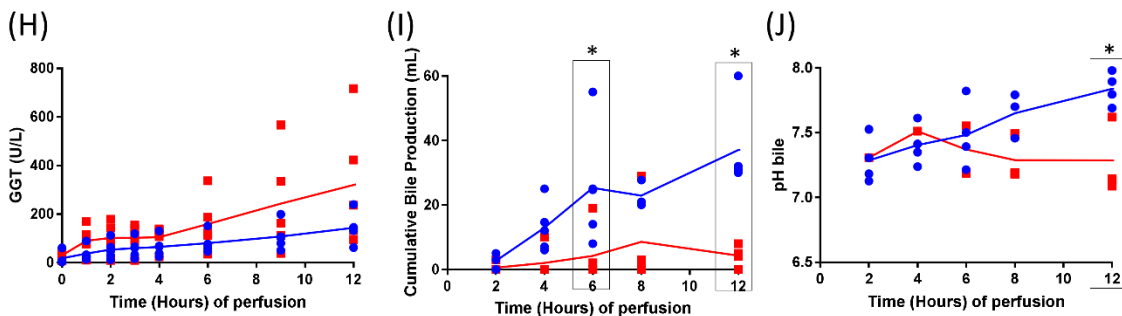
**Vascular parameters**



**Metabolic parameters**



**Biliary function and injury**



**Figure 7.1: Perfusion parameters.** Section 1 shows the vascular parameters of the livers during perfusion. The portal vein flow rate was higher in the defatted livers throughout the perfusion in comparison with the control group and this was associated with a faster decrease in vascular resistance (Panel B). Panel C shows the hepatic artery (HA) flow rate with a trend of higher values for the defatting group. This is potentially associated with the faster decrease in HA vascular resistance at the beginning of perfusion (Panel D). Metabolic parameters of the livers during machine perfusion were described in Section 2. Perfusate urea concentration increased significantly more in the defatting group in comparison with the controls that also had a rising trend (Panel E). Defatted livers metabolised perfusate lactate more effectively than controls reaching lower median concentrations (Panel F). Glucose levels peaked during the initial two hours of perfusion and then decreased steadily. In the control group figures were stable initially and then tend to increase towards the end of the perfusion (Panel G). Section 3 reports data from biliary function and injury. Gamma-glutamyl transferase (GGT) levels in the perfusate increased significantly more in the control group (Panel H). Concomitantly, treated livers produced significantly more bile throughout the perfusion (Panel I) with a higher bile-pH (Panel J). In all panels the dots represent individual organs at the time points and the line the median of the values for each group. Comparison between groups used the Mann-Whitney *U* test. Statistical significance at  $*p < 0.05$ .

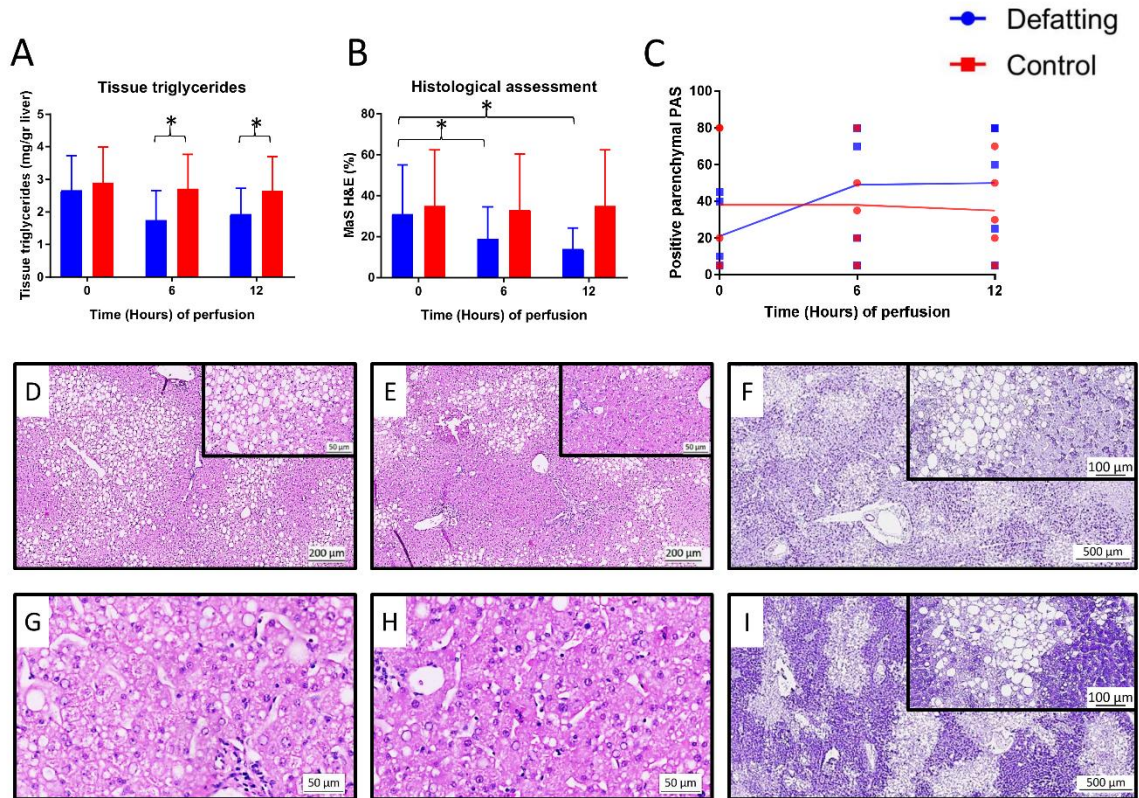
#### 7.4.2 Liver parenchyma histology

Each experimental group initially had 1 liver histologically classified as severely steatotic, 2 as moderately, 1 mildly and 1 non-steatotic. Changes between categories throughout the perfusion and the presence of MiS are presented in Table 7.2. Positive PAS staining of parenchymal area was constant along the perfusion for the control group whereas the defatting group presented with a trend of increase mainly on the initial 6 hours of perfusion (Figure 7.2).



**Figure 7.2: Tissue triglycerides content and histological steatosis assessment analysis.**

Tissue triglycerides content and steatosis histological assessment analysis



Tissue homogenate triglycerides were comparable at time 0 and then it decreased over 6 and 12 hours of perfusion in the defatting group reaching lower levels than the control group (Panel A). At the histological assessment of macrovesicular steatosis on haematoxylin and eosin staining (Panel B) a similar trend was observed. Panel C shows that defatted livers were more depleted of glycogen at time 0, however, their levels increased surpassing the control group already at 6 hours of perfusion. The positive areas for periodic-acid Schiff (PAS) were constant for the control group throughout the perfusion. Panel D, image from a histologically moderately steatotic liver that received the defatting cocktail and presented with a significant reduction in macrovesicular steatosis already at 6 hours of perfusion (Panel E). Panel G shows the presence of microvesicular steatosis in the same liver at time 0 and the change at 6 hours (Panel H). Panel F illustrates a mildly steatotic liver from the defatting group with an important depletion of glycogen stores replenishing its stores over 6 hours of perfusion (Panel I). In panels A and B, the bar represents the median and error bars the interquartile range. Panel C, the dots represent individual organs at the time points and the line the median of the values for each group. Comparison between groups used the Mann-Whitney U test. Statistical significance at  $*p < 0.05$ .

### **7.4.3 Lipid metabolism modulation and defatting**

T-TG levels dropped by 38% (23–51%) in the defatting group and 7% (3–10%) in the control group over 6 hours ( $p = 0.003$ ); and, 30% (21–35%) in the defatting versus 10% (6–13%) in the control over 12 hours ( $p = 0.002$ ) in comparison with time 0. MaS rate of defatted livers decreased by 40% (17–50%) over 6 hours and 50% (15–60%) over 12 hours, there were no changes in the control group ( $p = 0.02$  and  $p = 0.005$ , respectively) (Figure 7.2 and Table 7.2).

**Table 7.2: Steatosis assessment analysis.**

Group and case	Donor type	CIT (min.)	Macrovesicular steatosis			Microvesicular steatosis		
			Time 0	Time 6	Time 12	Time 0	Time 6	Time 12
Defat. 1	DBD	823	Moderate	Moderate	Mild	Present	Present	Present
Defat. 2	DCD	729	Moderate	Moderate	Mild	Present	Present	Present
Defat. 3	DBD	700	Mild	None	None	Present	Present	Present
Defat. 4	DBD	754	Severe	Moderate	Moderate	Present	Present	Present
Defat. 5	DCD	820	None	None	None	Present	Present	Present
Control 1	DBD	520	Mild	Mild	Mild	Present	Present	Present
Control 2	DCD	740	Moderate	Moderate	Moderate	Present	Present	Present
Control 3	DCD	800	None	None	None	Present	Present	Present
Control 4	DBD	723	Severe	Severe	Severe	Absent	Absent	Absent
Control 5	DBD	735	Moderate	Moderate	Moderate	Present	Present	Present
Steatosis rates variation analysis								
Parameter			Defatting ( <i>n</i> = 5)			Control ( <i>n</i> = 5)		<i>p</i> -value
Tissue triglycerides analysis (mg/gr liver)								
T-TG (Time 0)			2.7 (1.9–3.5)			3.3 (1.9–3.7)		0.84
T-TG (Time 6)			1.6 (0.9–2.6)			3.2 (1.7–3.6)		<b>0.03</b>
T-TG (Time 12)			2.0 (1.2–2.6)			3.1 (1.6–3.4)		<b>0.02</b>
ΔT-TG (Time 0 to 6)			38% (23–51)			7% (3–10)		<b>0.003</b>
ΔT-TG (Time 0 to 12)			30% (21–35)			10% (6–13)		<b>0.002</b>
Histological assessment analysis (Macrovesicular steatosis – MaS)								
MaS (Time 0)			31% (10–58)			40% (7–65)		0.82
MaS (Time 6)			15% (5–35)			30% (7–65)		0.31
MaS (Time 12)			15% (5–22)			40% (7–65)		0.13
ΔMas (Time 0 to 6)			40% (17–50)			0% (0–12)		<b>0.02</b>
ΔMas (Time 0 to 12)			50% (15–60)			0% (0–12)		<b>0.005</b>

Abbreviations: Defat.- Defatting group; DBD- Donation after brain death; DCD- Donation after circulatory death; CIT- Cold ischaemia time; T-TG- Tissue triglycerides. ΔT-TG and ΔMas are related to the variation in the figures between beginning and end of the period divided by the beginning. Severity of macrovesicular steatosis assessed according to the parenchymal are occupied by hepatocytes with the fatty changes: none < 5%, mild 5–30%, moderate 30–60% and severe > 60%.

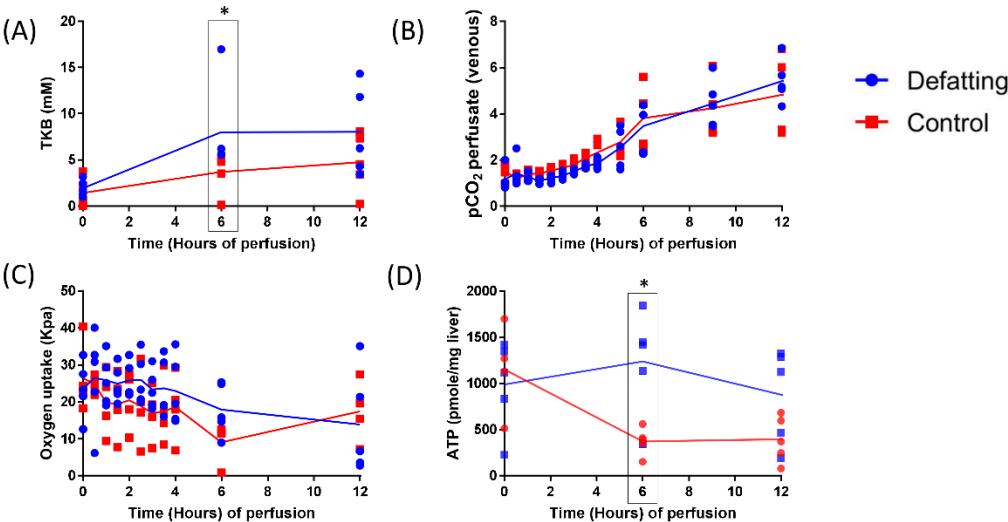
Ketogenesis was enhanced in the defatting group until 6-hour ( $p = 0.008$ ). ATP levels were similar at time 0 and then it increased significantly until 6 hours of perfusion for the defatting group and decreased for the control group ( $p = 0.01$  and  $p = 0.008$ , respectively) (Figure 7.3).

Median P-TG fell over the first 2 hours. Following this, it increased significantly until 6 hours in the defatting group ( $p = 0.04$ ) but not in the control ( $p = 0.59$ ). The increase in P-TG levels showed a correlation with the drop in T-TG between time points 0 to 6 hours ( $r = -0.58$ ,  $p = 0.08$ ) and 0 to 12 hours ( $r = -0.59$ ,  $p = 0.07$ ). There was no correlation between median P-TG levels and ALT concentrations in the perfusate ( $r = 0.18$ ,  $p = 0.65$ ).

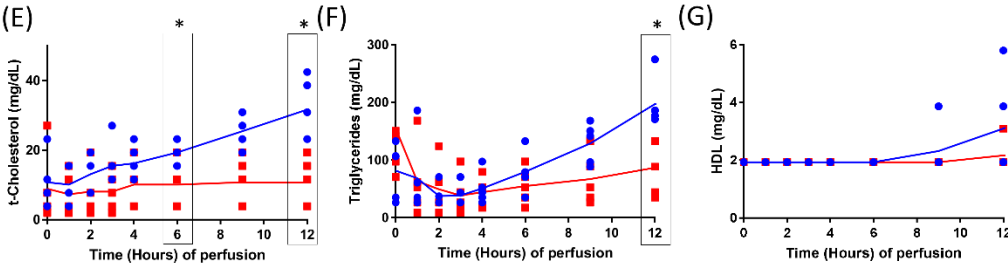
T-cholesterol levels followed the same pattern and they were higher in the defatting group at 6 ( $p = 0.02$ ) and 12 hours of perfusion ( $p = 0.003$ ). HDL-cholesterol was below the limit of detection of the laboratory until 6 hours of perfusion (1.9 mg/dL) starting to increase thereafter; however, did not achieve statistically significant difference at 12 hours of perfusion ( $p = 0.28$ ). Protein in-gel staining revealed that defatted livers had at 6-hour perfusion a 3-fold increase in the protein levels of acetyl-CoA synthetase, 1.4-fold in the Acyl-CoA oxidase 1 and 6-fold in CPT1A (Figure 7.3).

**Figure 7.3: Metabolic pathways for elimination of intracellular triglycerides.**

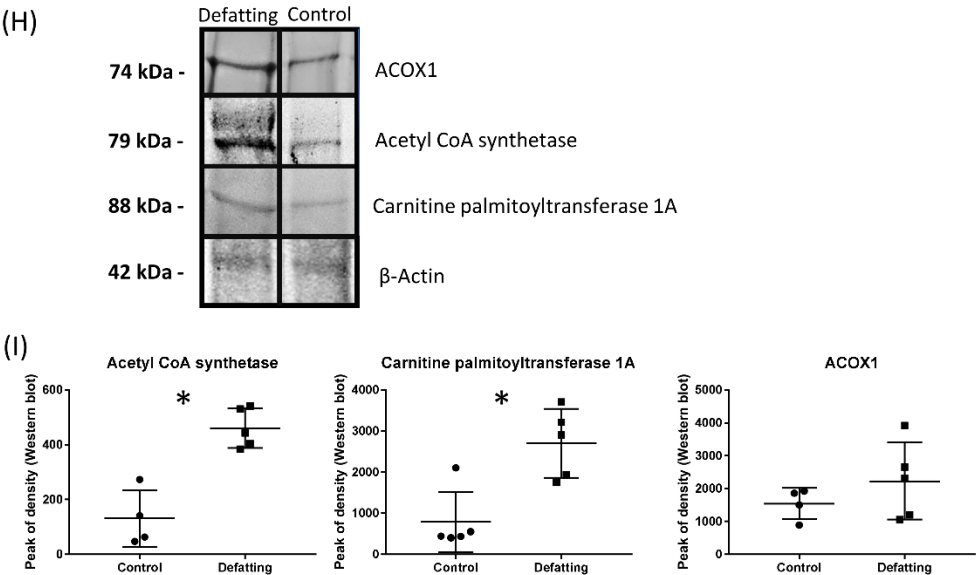
Mitochondrial oxidation of fatty acids



Exportation of fatty acids



Upregulation in lipid oxidation enzymes levels



**Figure 7.3: Metabolic pathways for elimination of intracellular triglycerides.** Enhanced mitochondrial oxidation of fatty acids (Series 1) results in increased ketogenesis (Panel A) throughout the perfusion, however, it was more pronounced on the treatment group. Mitochondrial respiration assessed by  $p\text{CO}_2$  production increased steadily on both experimental groups (Panel B) and the oxygen uptake was higher for the initial 3 hours in the defatting group and then decreased slightly (Panel C). This was associated with increased ATP replenishing within the initial 6 hours in the defatting group and then decreased steadily maintaining higher concentrations than the control group. In addition, there was an increase in exportation of fatty acids (Section 2). The levels of total cholesterol in the perfusate increased faster in defatted livers (Panel E) as the triglycerides' levels (Panel F). HDL cholesterol levels were below the detection range and start to increase slowly after 6 hours of perfusion (Panel G). In-gel fluorescent protein stain normalised to the total protein was performed for acetyl CoA synthetase, carnitine palmitoyltransferase form 1A (CPT1A) and peroxisomal acyl-coenzyme A oxidase 1 (ACOX1). Experiments were performed  $n = 5$  and representative figures are presented in Panel H. The peak of intensity in densitometric analysis at the specific protein band was compared between the groups (Panel I). In all panels the dots represent individual organs at the time points and the line the median of the values for each group. Comparison between groups used the Mann-Whitney U test. Statistical significance at  $*p < 0.05$ .

#### 7.4.4 Liver viability, hepatocellular metabolic functional parameters and biomarkers

Lactate levels were comparable at time 0 and then they decreased significantly until 12-hours of perfusion in the treatment group, but not for the control group ( $p = 0.04$  and  $0.22$ , respectively). Dynamic changes in the lactate metabolism, as assessed by the AUC, showed that, considering both groups, smaller lactate-AUC was associated with higher total perfusion flows ( $r = -0.84$ ,  $p = 0.002$ ), increased urea production ( $r = -0.73$ ,  $p = 0.02$ ), higher bile pH at time 6-hour ( $r = -0.88$ ,  $p = 0.02$ ), increased release of P-TG ( $r = -0.90$ ,  $p < 0.001$ ), lower expression of 4-HNE ( $r = 0.62$ ,  $p = 0.05$ ) and a trend for higher bile production ( $r = -0.58$ ,  $p = 0.08$ ). The defatting group demonstrated a trend of shorter time to peak lactate and time to achieve lactate  $< 2.5$  mmol/L, which were correlated with positive metabolic factors. Further details are provided in Figure S7.1.

All treated livers reached our viability criteria and therefore would be considered transplantable based solely upon this criterion. From the control group only 2 organs recovered high enough

functional parameters to be considered transplantable ( $p = 0.04$ ). Achievements of the parameters of the criteria are presented in Table S7.3.

Defatted livers produced more urea over the initial 6 hours ( $p = 0.03$ ). There was a strong negative correlation between lactate and urea levels at 6-hour ( $r = -0.82$ ,  $p = 0.004$ ) and 12 hours ( $r = -0.77$ ,  $p = 0.009$ ). Increased P-TG correlated with an increased production of urea at 6-hour ( $r = 0.87$ ,  $p = 0.001$ ) and 12-hour ( $r = 0.63$ ,  $p = 0.05$ ). Median perfusate glucose increased from time 0 to 2-hours in the defatting group ( $p = 0.10$ ) and then decreased towards 12-hour ( $p = 0.08$ ). Those levels were constant for the control group. Metabolic parameters of the livers are presented in Figure 7.1. Electrolytes and other biochemistry parameters are presented in Figure S7.2.

The viable livers from the control group released less t-cholesterol (17.4 [15.5–19.3] vs. 30.9 [23.2–38.7] mg/dL,  $p = 0.02$ ) and P-TG (110.7 [88.6–132.9] vs. 177.1 [177.1–186.0] mg/dL,  $p = 0.048$ ) at 12-hour, produced less ATP (400 [390–410] vs. 1417 [1138–1453] pmol/mg,  $p = 0.03$ ) and developed higher expression of 4-HNE (IRS 2 [2–2] vs. IRS 1 [1–1],  $p = 0.02$ ) at time 6-hour in comparison with defatted livers.

#### **7.4.5 Biliary injury and function**

Cumulative bile production was higher for the defatting group at the 6-hour ( $p = 0.03$ ) and 12-hour perfusion time points ( $p = 0.008$ ). There was no statistically significant correlation between t-cholesterol in the perfusate and cumulative bile production at 6 hours ( $r = 0.59$ ,  $p = 0.07$ ) or 12 hours of NMP ( $r = 0.59$ ,  $p = 0.07$ ).

Bile quality, as assessed by bile's pH measured at 12 hours, was also higher in the defatting group (7.84 [7.71–7.95] vs. 7.34 [7.10–7.60],  $p = 0.03$ ). GGT levels in the perfusate increased

significantly from time 0 to 12 hours of perfusion in the control group ( $p = 0.04$ ). Details are presented in Figure 7.1.

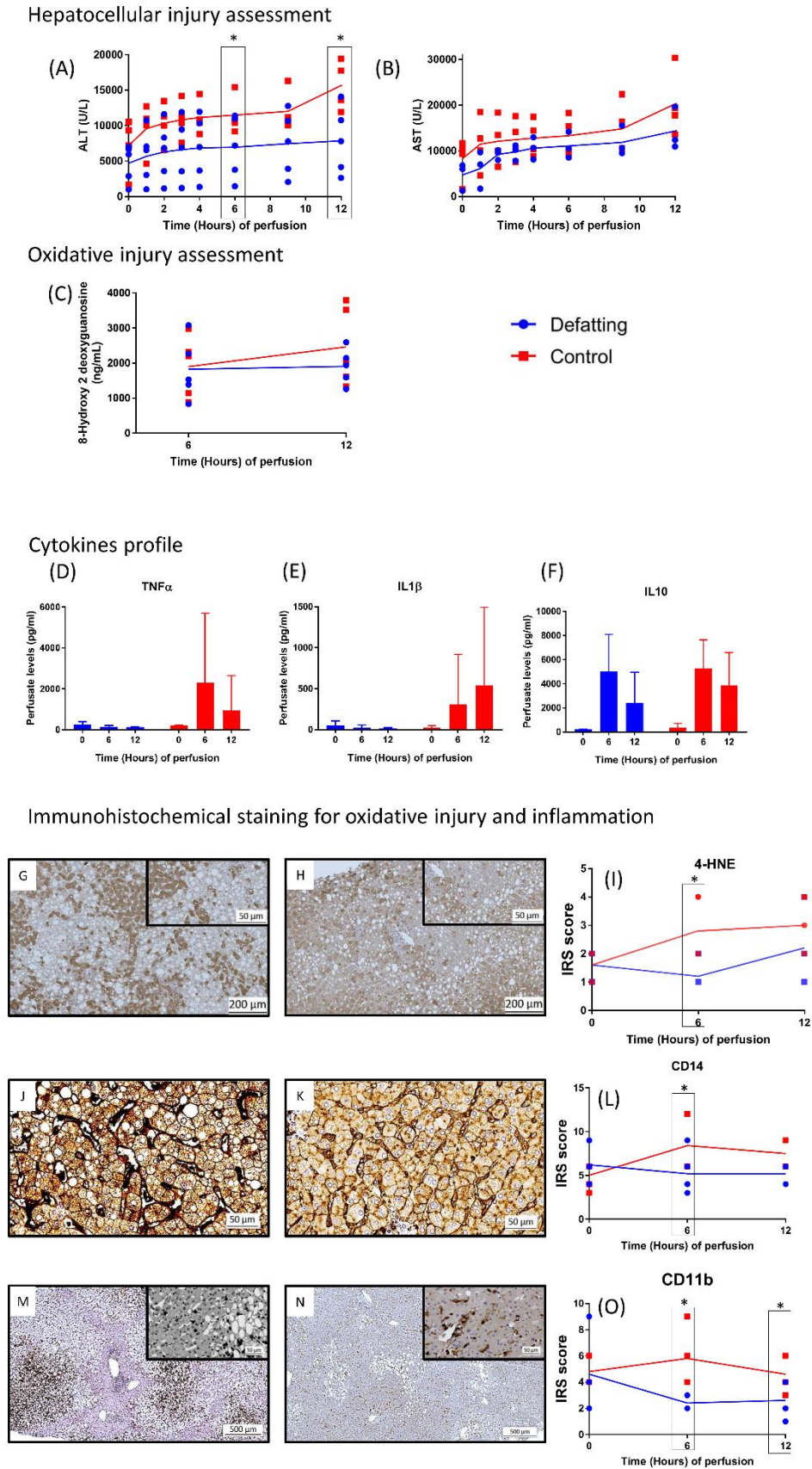
#### **7.4.6 Hepatocellular injury, oxidative damage and activation of immune cells**

Perfusate ALT levels were lower in the defatted livers at 12-hour perfusion ( $p = 0.049$ ) (Figure 7.4). Perfusate 8-OHdG levels were similar at the 6-hour time point followed by a general increase until 12 hours in the control group and a stabilisation of levels in the defatting group ( $p = 0.10$ ). Immunohistochemical analysis for 4-HNE demonstrated similar initial levels at the beginning of the perfusion followed by an increase in the control group and a decrease in the defatting at 6 hours ( $p = 0.02$ ). There was a strong negative correlation between P-TG and 4-HNE expression ( $r = -0.87$ ,  $p = 0.001$ ) at 6-hour of perfusion.

The activation of the Kupffer cells and neutrophils in liver tissue, as assessed by immunohistochemical analysis (CD14 and CD11b, respectively), was similar between groups at time 0 and then it increased for the control group reaching higher scores at 12 hours ( $p = 0.046$  and  $p = 0.045$ , respectively). The cytokine profile showed a decrease in the perfusate levels of the inflammatory TNF- $\alpha$  from time 0 to 6, and 12 hours in the defatting group. IL-1 $\beta$  also decreased over the time-course of the perfusions in the treatment group. For both markers, the levels in the control were either flat or increased. The anti-inflammatory IL-10 increased in both groups from the beginning until time 6 hours and then it decreased faster in the defatting group (Figure 7.4).



**Figure 7.4 Assessment of hepatocellular, oxidative injury and activation of the immune cells.**



**Figure 7.4: Assessment of hepatocellular, oxidative injury and activation of the immune cells.** Alanine aminotransferase (ALT) levels released in the perfusate were investigated as a marker of hepatocellular injury (Section 1). Levels flattened along the perfusion in the defatting group and reached higher figures in the control group (Panel A). Aspartate transaminase (AST) levels followed a similar trend (Panel B). Markers of oxidative injury were also explored (Section 2). Nuclear cells damage assessed by 8-HOdG released in the perfusate showed stable levels in the defatting group and a trend to increase in the control group (Panel C). The cytokines' profile (Series 3) was investigated and perfusate concentration of the pro-inflammatory cytokines TNF $\alpha$  (Panel D) and IL-1 $\beta$  (Panel E) decreased along the perfusion in the defatting group and increased in the group. The anti-inflammatory IL-10 increased in both groups at 6 hours and then it decreased faster in the defatted livers (Panel F). Immunohistochemical analysis was performed for markers of oxidative injury and activation of an inflammatory response. Panel G shows a moderate reaction of the staining committing 10–50% of the hepatocytes as assessed by the immunoreactive score (IRS). After 6 hours of defatting the figure shows a predominantly mild staining reaction in 10–50% of the hepatocytes (Panel H). The trend for each experimental group along the perfusion is presented in Panel I. In accordance, tissue expression of CD-14 (Panels J and K) and CD-11b (Panels M and N) for the defatting group decreased from the beginning to time 6 hours of perfusion. Panel L represents the changes over time for CD-14 and Panel O the changes in tissue expression of CD-11b. Scales are provided on the bottom of each figure. In all panels, the dots represent individual organs at the time points and the line the median of the values for each group. Comparison between groups used the Mann-Whitney U test. Statistical significance at  $*p < 0.05$ .

## 7.5 Discussion

Steatosis has become the leading reason for declining donor livers for transplantation which, in turn, is worsening the growing discrepancy between organ availability and the increasing transplant waiting lists (12, 13). We have shown that the delivery of a pharmacological intervention during NMP was able to decrease the fat content of whole steatotic human livers within 6 hours. This effect was driven by enhanced lipid metabolism as increased oxidation of lipids and export to the perfusate. Importantly, this enhanced lipid metabolism increased the metabolic support to all organs improving its functional recovery. It was associated with enhanced mitochondrial functioning, increased vascular flows, reduced markers of hepatocellular injury and inflammation with improved biliary function. These parameters are suggested as indicatives of transplantability of extended criteria donor (ECD) organs during NMP according to the evidence available to date (14, 15). Therefore, manipulation of the

hepatic lipid metabolism during NMP may defat steatotic organs and increase their functional recovery potentially halting the harmful effects of reperfusion injury.

Previous *in vitro* studies with fat-laden rat hepatocytes and hepatoblastoma cells demonstrated the feasibility of defatting cells with a combination of drugs over 48 hours (6, 16, 17). Thereafter, this defatting cocktail was delivered to whole steatotic rat livers during NMP and the authors showed a decrease in tissue-TG of 50% within 3 hours (6). Interestingly a decrease of 30% could be obtained with NMP alone over the same period (6). However, a recent study reporting results of 24-hours perfusion of steatotic human livers could not replicate the same results with NMP alone (5). This raised concerns about variability in response between species and that the effectiveness of the drugs could be compromised after the inherent ischaemic injury during organ retrieval.

A description of the detailed pharmacological effects of the drugs used is out of the scope of this manuscript, however it can be found within a comprehensive review about the subject (18). Briefly the drugs increase intracellular lipolysis releasing FAs and glycerol within the cells and induce the transcription of enzymes involved in its catabolism in the mitochondria and peroxisomes. Hence metabolization of lipid stores not only decreases its intracellular content, but also supports cell metabolism enhancing the recovery of the function of the organs. Importantly, these cellular metabolic pathways are not dependent on insulin receptors and therefore occurrence of insulin resistance, common in non-alcoholic fatty liver disease, should not affect the process. Our group recently reported, for the first time, the effectiveness *in vitro* of these pharmacological agents in defatting primary human hepatocytes made fatty by supplementation with fatty acids (19). Apart from being non-toxic to these cells, there was a decrease of 35% in intracellular tissue triglyceride over 48 hours of incubation. These agents

were also shown for the first time not be toxic to non-parenchymal liver cells (intrahepatic endothelial cells and cholangiocytes) (19).

In this study we delivered a defatting cocktail to human livers, discarded for transplantation due to the surgical macroscopic assessment of steatosis. All livers across both groups had uniformly extended periods of CIT. We have found that NMP supplemented with the defatting cocktail was able to improve lipid metabolism, decreasing significantly tissue-TG by 38% over a period of 6 hours whereas NMP alone reduced it by 7% over the same time. Histologically, it corresponded to a decrease of 40% in MaS and 0%, respectively. This defatting effect was associated with an enhanced mitochondrial function ( $\beta$ -oxidation of FAs, mitochondrial respiration [higher oxygen uptake], ketogenesis, augmented urea cycle and Krebs cycle activity with higher ATP synthesis) and increased exportation of intracellular-TG as lipoproteins in the perfusate. Mechanistically, it was represented by upregulation in the levels of key enzymes involved in the intracellular lipid metabolism in comparison with control livers. Beneficial effects were also seen in glucose metabolism, where defatted livers released more glucose in the perfusate during the initial hours of perfusion. This enhanced gluconeogenesis is likely to be related to an increased availability of glycerol because of the augmented breakdown of lipids with higher production of glucose-6-phosphate. Glucose-6-phosphate, in turn, will generate the glucose released in the perfusate and activate the enzyme glycogen synthase promoting glycogenesis, in accordance with our findings (20).

In addition, modulation of the lipid metabolism halted hepatocellular injury as assessed by diminished release of ALT in the perfusate. Tissue damage during reperfusion is primarily related to mitochondrial dysfunction with reactive oxygen species (ROS) production, oxidative stress and concomitant pro-inflammatory response. Accordingly, treated livers developed lower cellular DNA damage (8-OHdG) by the end of the perfusion, lower rates of lipid peroxidation

(4-HNE), production of inflammatory cytokines and activation of immune cells (CD-14, CD-11b). The former could also have resulted from direct effects of the defatting constituents, such as PPARs (21, 22). Our findings corroborate with the previous *in vitro* studies, where delivering the cocktail to fat-laden rat hepatocytes diminished ROS concentration and cellular injury, as assessed by the cytosolic lactate dehydrogenase release into media (16).

Ischaemic cholangiopathy following DCD liver transplantation is of great clinical concern and identification of high-risk donor organs during NMP, prior to transplantation, would be a useful clinical tool (23). Watson *et al.* 2018 described that bile-pH < 7.5 during NMP is suggestive of biliary tree necrosis and can increase the risk of ischaemic cholangiopathy post-transplantation (15). Contrary to this, treated livers demonstrated bile-pH levels higher than 7.5 while the converse was seen in control livers. Importantly, there was no correlation between greater bile production and the increase in t-cholesterol in the perfusate. This suggests a beneficial effect of the cocktail itself on the biliary system. The mechanism involved needs further investigation. However, the protective role of defatting lowering oxidative injury and tissue damage can be potentially correlated.

In *in vitro* studies the defatting cocktail was also associated with a higher resistance to IRI, as demonstrated by improved hepatocyte viability (16, 24, 25). In our study pharmacological modulation of the lipid metabolism of donor livers during NMP reduced lipid content and improved their functional recovery. Urea is produced by the liver and results from an enzymatic reaction involving carbon dioxide and the ammonia derived from the deamination of proteins in the liver. The increased level of urea suggests increased urea synthesis in defatted livers (17, 26). Our previously published viability criteria assert that lactate levels < 2.5 mmol/L within 4 hours is a major criterion for safe transplantation of ECD donor livers. However, it also considers other parameters of liver metabolism (10, 14). We have performed a separate analysis

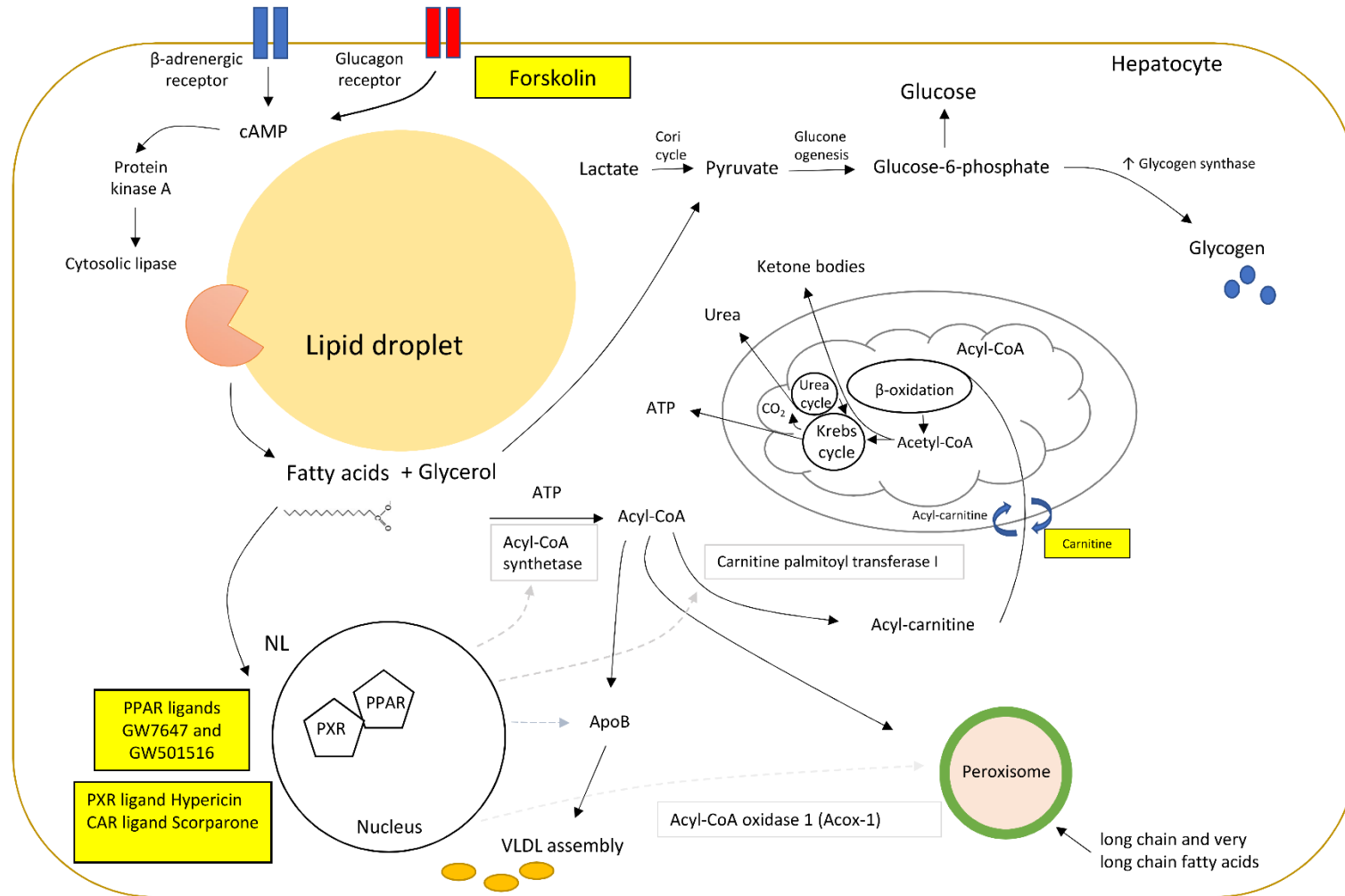
of the dynamic changes in lactate concentration over time; it showed a strong correlation between lactate metabolism kinetics (lower AUC, shorter time to peak lactate and the clearance from peak to 2.5 mmol/L) and other parameters of appropriate liver metabolism. These findings reinforce the significance of this marker in the context of defatting. The Cambridge group has advocated the use of transaminases, bile production and bile quality as appropriate markers of organ viability (15). The discussion about optimal viability criteria during NMP is out of the scope of this paper, and whether the same criteria or machine perfusion technique are applicable for all organs (e.g. DBD, DCD or defatted livers) is also another topic for discussion. However, defatted livers fulfilled all those criteria currently considered as indicative of adequate liver metabolism and, hence, would potentially be deemed transplantable.

The results obtained from employing our study protocol have shown that pharmacological targeting of lipid metabolism can defat steatotic human livers and improve their metabolic function within the relatively short timeframe of 6 hours. Beyond this time point, all the benefits observed with the defatting protocol appeared to be sustained. This observation suggests that the drugs had already been metabolised at the 6-hour perfusion mark. At this point, either a second bolus of the cocktail could be considered to amplify its effects, or the aim would be considered achieved and the organ potentially used for transplantation. Another point of discussion is the use of filters to remove solubilised lipids from the perfusate. It intends potentially to prevent lipotoxicity. However, in the context of NMP and defatting we have not seen toxic effects of the FAs in organ functioning, tissue damage or inflammatory response. Further studies will be needed to investigate its potential advantages.

Banan *et al.* showed a reduction of 10% in MaS over 8 hours of NMP for a discarded steatotic donor human liver with 80% MaS and negligible reduction for a liver with 30% MaS, supplementing the perfusate with 10 mM of L-carnitine. However, the variability between the

two organs reported and the lack of a control group limits the interpretation of the results of this study. This is the first study designed specifically to deliver a defatting cocktail of drugs to steatotic human livers during NMP and assess its impact on lipid metabolism. The increased breakdown of intracellular triglycerides was associated with signs of improved mitochondrial oxidative function (urea cycle, Krebs cycle, ketogenesis, ATP synthesis) that supported other metabolic process, as glycogenesis, gluconeogenesis, and alleviate ROS induced oxidative stress and pro-inflammatory response halting hepatocellular injury (a diagrammatic summary is provided in Figure 7.5). Defatted livers developed higher vascular flows, lower vascular resistances that correlated with the higher solubilisation of triglycerides in the perfusate and enhanced biliary function. Importantly, all these observations were made within a scenario of clinical organ donation encompassing livers originating from different donors (DCD and DBD) exposed to extended periods of CIT before receiving treatment.

**Figure 7.5: Suggested relationship between the defatting cocktail and intracellular lipolytic metabolic pathways.**





**Figure 7.5: A suggested relationship between the defatting cocktail and intracellular lipolytic metabolic pathways.** Forskolin activating glucagon membrane receptors stimulates the cyclic adenosine monophosphate (cAMP) – protein kinase A pathway and cytoplasmic lipases are attracted to the surface of lipid droplets. Glycerol and fatty acids (FA) will then be released serving not only as substrates for the cell metabolism but also as ligands to nuclear receptors (peroxisome proliferator receptor [PPAR] and liver X receptors [LXR]) increasing the transcription of enzymes involved in the catabolism of FFAs in the mitochondria and peroxisome. Diverse cocktail drugs (GW7647, GW501516, hypericin, scorparone) also acts as ligands to other nuclear receptors (pregnane X receptors and androstane receptors) boosting the transcription of key enzymes. Cytosolic fatty acid reacts with adenosine triphosphate (ATP) molecules producing fatty acyl-CoA. Acyl-CoA in turn reacts with apolipoprotein B to generate lipoproteins to be exported from the cell and/or reacts with the hydroxyl group of carnitine via carnitine palmitoyltransferase I. Acyl-carnitine is transported inside the mitochondria by a carnitine-acyl-CoA transferase and a carnitine is transferred outside. Acyl-CoA is processed by  $\beta$ -oxidation. The acetyl-CoA produced then will follow to ketogenesis or for complete oxidation via the Krebs cycle and the electron transport chain with the production of ATP. Increased production of aspartate and carbon dioxide (CO<sub>2</sub>) stimulate the urea cycle increasing the production of urea. Alternatively, long and very-long-chain FFAs are also oxidized in the peroxisomes. Glycerol is a gluconeogenic precursor, it is converted to pyruvate producing glucose-6-phosphate through the gluconeogenesis pathway. Glucose-6-phosphate will be released as glucose in the perfusate and stimulate the enzyme glycogen synthase increasing the production of glycogen. The squares contain the specific enzymes that have an upregulation in the transcription as a consequence of this stimulus. Drugs and supplement used in the cocktail are presented in yellow squares.

Although our study has shown that *ex situ* pharmacological modulation of lipid metabolism of donor human livers enhances its function, we have not proven that they are transplantable. This clearly demands a pilot study to assess the defatting protocol in clinical transplantation. In the United Kingdom histological assessment of donor liver steatosis is not performed routinely, and livers are discarded solely based on a surgical macroscopic assessment of steatosis. As a consequence, we had various grades of steatosis, assessed at the histological level, in the groups, and even two livers with less than 5% MaS. However, they were equally distributed between groups. Moreover, despite controlling for donor type, CIT and the groups having livers with similar degrees of steatosis, some other unfavourable factors were more predominant in the control group (higher GGT peak, higher liver weight, the oldest donor in the cohort). Small differences between groups is an intrinsic limitation of research with discarded human livers, however, the use of human livers in research has the advantage of eliminating variability between species, the latter being a limitation of animal model studies. All livers had prolonged

CIT, this together with steatosis, and other donor factors, explain the poor rescue of control livers based on metabolic parameters.

## **7.6 Conclusion**

In conclusion, we have shown that pharmacological modulation of lipid metabolism during NMP can promote defatting of whole human steatotic livers within 6 hours. More importantly, the enhanced lipid metabolism improved the metabolic status of the organs, their functional recovery, decreased vascular resistance and reduced the expression of markers of reperfusion injury. Those findings support further clinical investigations and open a window of opportunity for better utilisation of steatotic donor livers.

## **7.7 Appendices**

### **7.7.1 Supplementary Material and Methods**

#### **7.7.1.1 Sampling protocol**

##### **7.7.1.1.1 Liver biopsies**

Core needle liver biopsies using disposable modified Menghini needle (15G) were taken before commencing the perfusion, at time points 6-hour and 12-hour of perfusion for all livers. Two biopsies, not more than 1 cm from each other, were taken at each time point. One was stored in formalin and the other immediately snap-frozen in liquid nitrogen for further analysis.

##### **7.7.1.1.2 Perfusate samples**

The perfusate was sampled every 30 minutes throughout the first 4 hours of perfusion and then at 6-hour, 9-hour and 12-hour of perfusion for all livers.

At each time point approximately 1 millilitre (mL) of perfusate was analysed immediately using the Cobas b 221 point of care system (Roche Diagnostics, USA) blood gas analyser. The partial pressures of oxygen (O<sub>2</sub>), and carbon dioxide (CO<sub>2</sub>), pH, concentrations of base excess, bicarbonate, O<sub>2</sub> saturation, haemoglobin, haematocrit, sodium, potassium, chloride, calcium, glucose and lactate were measured. Four mL of perfusate were then freshly collected in serum separators tubes for biochemistry analysis at the hospital clinical laboratories. Six millilitres were centrifuged at 10,000 rotations per minute for two minutes and the supernatant collected and snapped-frozen for further analysis.

#### **7.7.1.1.3 Bile samples**

Bile production was collected into a Jackson-Pratt drain accoupled to the pipe connected with the bile duct. The drain was emptied at 2-hour, 4-hour, 6-hour, 8-hour and 12 hours of perfusion for all livers.

At each time point the total volume of bile produced was recorded and the bile analysed using the Cobas b 221 point of care system (Roche Diagnostics, USA) blood gas analyser. Four 4 mL were collected into cryovials and snapped-frozen for further analysis.

#### **7.7.1.2 Data collection protocol**

Parameters for the arterial and venous perfusion units (flows, pressure, resistance and temperature) showed on the device's display were registered every 30 minutes throughout the first 4 hours of perfusion and then at 6-hour, 9-hour and 12-hour of perfusion for all livers.

#### **7.7.1.3 Fluorescent protein gel stain**

Equal amounts of frozen liver tissue were homogenised in lysing buffer solution (Tris, 2% SDS [sodium dodecyl sulfate], 2 mM EDTA [Ethylenediaminetetraacetic acid], pH = 9.0) at 100 mg/mL using the Precellys 24 tissue homogeneiser (Bertin instruments, Montigny-le-Bretonneux France). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (23225; Thermo Scientific Pierce, Waltham, MA, USA) and final concentration equalised to 10 mg/mL. Samples were diluted in a 1:1 rate with 2x Laemmli sample loading buffer (1610737; Bio-Rad, Hercules, CA, USA) 5% 2-mercaptoethanol. Thereafter 20 µL of sample total protein were loaded in each well of a polyacrylamide gel (Sodium dodecyl sulfate-polyacrylamide gel - SDS-PAGE) (4561045; Bio-Rad, Hercules, CA, USA) on the Criterion Dodeca gel apparatus (Bio-Rad) and protein bands were separated by electrophoresis at 200V for 30 minutes in running buffer (1610732; Bio-Rad). Precision Plus Protein™ WesternC™

Blotting Standards (Bio-Rad) was used as a protein ladder. An alcohol solution (50% isopropanol, 5% acetic acid prepared in ultrapure water), was used to fix protein bands on the matrix and the gel was incubated for 1 hour with the primary antibody (see antibody list). Following the gel was washed in PBS (Phosphate-buffered saline) plus 0.1% Tween 20 and then incubated with the secondary fluorescent antibody for an additional 1 hour (see antibody list).  $\beta$ -Actin antibody was used as a loading control to confirm that samples were equally loaded and the effectiveness of the electrophoresis and imaged using a secondary fluorescent antibody. Gels were imaged with the ChemiDoc MP imager (Bio-Rad, Hercules, CA, USA) using specific protocols for the antibodies used based upon their excitation and emission specifications. Images were analysed using the ImageLab software version 6.0 (Bio-Rad). Background was subtracted from all lanes and the peak of intensity in densitometric analysis at the specific protein band compared between the groups.

#### **7.7.1.4 Immunohistochemical staining**

Immunohistochemistry was performed on paraffin-embedded tissue sections of 4  $\mu$ m thickness using the ImmPRESS<sup>TM</sup> Excel Staining kit (MP-7601 and MP-7602; Vector Laboratories, Burlingame, CA, USA). The sections were deparaffinized in 3 baths of xylene for 3 minutes and rehydrated in a similar series of ethanol. Microwaving antigen retrieval was performed in Tris-based solution (H-3301; Vector Laboratories), pH 9, for 25 min. The sections were washed using Dako wash buffer (S3006; Dako Agilent, Santa Clara, CA, USA). Quenching of endogenous peroxidase activity was done via incubation in BLOXALL<sup>TM</sup> Blocking Solution (SP-6000; Vector laboratories) for 10 minutes and blocked with 1x casein for 10 minutes (SP-5020; Vector Laboratories). Sections were then incubated with the primary antibodies diluted in 1x casein (see antibody list for specific dilutions used) for 1 hour. After a second wash tissue sections were incubated with an amplifier antibody (goat anti-mouse IgG or goat anti-rabbit

IgG) ready-to-use (Vector laboratories) for 15 minutes. A new wash was performed, and the tissue sections incubated with ImmPRESS™ Excel reagent for 30 minutes. The positive immuno-reactivity was revealed by incubation with a mixture of ImmPACT™ DAB EqV Reagent 1 with Reagent 2 (Vector laboratories). Tissue sections were counterstained with Mayer's haematoxylin. Finally, sections were dehydrated in a series of ethanol and xylene. No primary negative controls were obtained by replacing primary antibody for equal volume of goat serum and isotype negative controls by replacing the primary antibody for an isotype control antibody.

All sections were wholly digitalized using the slide scanner Axio Scan.Z1 (Carl Zeiss, Oberkochen, Germany) and the images for the study selected using the ZEN image analysis software.

#### **7.7.1.5 Antibodies list**

##### **7.7.1.5.1 Immunohistochemical staining**

- Anti-4-hydroxynonenal (4-HNE) antibody (ab46545; Abcam, Cambridge, MA, USA) rabbit polyclonal IgG at 1:200 dilution.
- Anti-Cluster of differentiation 14 (CD14) antibody (ab36595; Abcam, Cambridge, MA, USA) mouse monoclonal at 1:500 dilution.
- Anti-Cluster of differentiation 11b (CD11b) antibody (ab52478; Abcam, Cambridge, MA, USA) rabbit monoclonal at 1:200 dilution.
- Rabbit IgG isotype control antibody (ab172730; Abcam, Cambridge, MA, USA) monoclonal at 1:200 dilution.
- Mouse IgG isotype control antibody, clone MG3-35 (catalogue number 401302; BioLegend, San Diego, CA, USA) unspecific at 1:500 dilution.

#### **7.7.1.5.2 Protein gel staining**

##### **7.7.1.5.2.1 Primary antibodies**

- Anti-Acetyl CoA synthetase antibody (ab133664; Abcam, Cambridge, MA, USA) rabbit monoclonal IgG at 1:1,000 dilution.
- Anti-ACOX1 antibody (ab184032; Abcam, Cambridge, MA, USA) rabbit monoclonal IgG at 1:1,000 dilution.
- Anti-CPT1A antibody (ab83862; Abcam, Cambridge, MA, USA) rabbit polyclonal IgG at 1:1,000 dilution.
- Anti- $\beta$ -Actin antibody (A5441; Sigma-Aldrich, St. Louis, MO., USA) mouse monoclonal IgG at 1:1,000 dilution.

##### **7.7.1.5.2 Secondary antibodies**

- Sheep anti Rabbit IgG:DyLight<sup>®</sup>488 (STAR36D488GA; Bio-Rad, Hercules, CA, USA) polyclonal IgG at 1:1,000 dilution.
- Goat anti Mouse IgG:DyLight<sup>®</sup>800 (STAR117D800GA; Bio-Rad, Hercules, CA, USA) polyclonal IgG at 1:1,000 dilution.

##### **7.7.1.6 Reagents list**

All drugs employed in the experiments were bought from Sigma-Aldrich, St. Louis, MO., USA. The corresponding catalogue numbers are: Forskolin-F6886; GW7647-G6793; Hypericin-56690; Scopolamine-254886; GW501516-SML1491; Visfatin-SRP4908; and, L-carnitine-C0283. General laboratory consumables used were mostly bought from Sigma-Aldrich.

## 7.7.2 Supplementary Results

### 7.7.2.1 Table S7.1: Normothermic machine perfusion fluid constitution.

**Table S7.1: Normothermic machine perfusion fluid constitution.**

3 units of group O Rhesus-negative donor packed red blood cells
1000 mL of 5% w/v human albumin solution (Alburex 5, CSL Behring GmbH, Germany)
10,000 IU heparin (Wockhardt, UK)
30 mL sodium bicarbonate 8.4% (B. Braun Medical Limited, UK)
10 mL calcium gluconate 10%
500 mg vancomycin (Wockhardt, UK)
60 mg gentamicin (Cidomycin, Sanofi, UK)
50 mL of 10% v/v Aminoplasma (B. Braun Medical Limited, UK)
0.2 mL Cernevit (Baxter Healthcare Ltd., UK)
0.1 mg phytomenadione (Konakion, Roche Products Ltd, UK)
Epoprostenol (Flolan, GlaxoSmithKline, UK, 2 µg/mL) continuous infusion at 4 mL/hour

**Abbreviations:** UK- United Kingdom.



**7.7.2.2 Table S7.2: Donor demographics, liver features and perfusion parameters.**

Liver number	CONTROL 1	CONTROL 2	CONTROL 3	CONTROL 4	CONTROL 5	DEFAT 1	DEFAT 2	DEFAT 3	DEFAT 4	DEFAT 5
<b>Donor information</b>										
Age	81	48	49	52	41	51	69	54	52	44
Donor type	DBD	DCD	DCD	DBD	DBD	DBD	DCD	DBD	DBD	DCD
Gender	Male	Male	Male	Female	Female	Male	Male	Male	Female	Female
Height (cm)	173	170	203	172	165	174	193	175	169	155
Body weight (kg)	76	95	115	75	95	81	101	98	86	70
Body mass index (kg/m <sup>2</sup> )	25	33	28	25	35	37	27	32	30	29
Donor risk index	2.08	2.00	2.09	1.99	1.67	2.00	3.02	1.83	2.04	2.58
UK donor liver index	1.52	2.01	1.28	1.09	0.81	1.00	1.77	1.15	0.90	1.78
ET donor risk index	2.02	2.01	2.59	2.41	1.96	1.76	3.41	1.85	1.75	2.82
Peak ALT (IU/L)	39	74	177	104	335	76	67	110	30	65
Peak GGT (IU/L)	31	--	137	402	355	31	71	150	38	269
Days on ventilator	1	5	4	3	2	2	2	3	2	4
Co-morbidities and risk history	Hypertension, light drinker	Heavy drinker	Light drinker	Heavy drinker, depression	Hypertension	Diabetes (type 2)	Diabetes (type 2), moderate drinker	Diabetes (type 2), Smoker	Hypothyroidism	Depression, ulcerative colitis
Cause of death	Trauma	Hypoxic brain damage	Hypoxic brain damage	Intracranial haemorrhage	Hypoxic brain damage	Intracranial haemorrhage	Intracranial haemorrhage	Intracranial haemorrhage	Intracranial haemorrhage	Hypoxic brain damage
<b>Organ information</b>										
Liver weight (grams)	1699	1852	2139	2130	2317	1768	2330	2394	1874	1694
Donor warm ischaemic time (minutes)	--	17	15	--	--	--	13	--	--	14
Cold ischaemia time (minutes)	520	740	800	723	735	823	729	700	754	820
Reason for clinical rejection	Steatosis	Steatosis	Steatosis (severe on biopsy)	Steatosis	Steatosis	Steatosis	Steatosis	Steatosis	Steatosis (severe on biopsy)	Steatosis (severe on biopsy)
<b>Perfusion characteristics</b>										
Lactate (mmol/L)										
Highest	14.7	10.3	13.9	> 20.0	17.7	11.6	11.0	9.9	12.7	20.0
Lowest	1.6	0.8	0.7	11.8	11.6	1.9	0.9	0.2	1.8	0.4
Last	1.9	2.7	1.0	> 20.0	13.4	1.9	0.9	0.3	1.8	0.4
Total Bile production (mL)	6.0	40.0	8.0	0	38.0	70	40.0	31.0	40.0	30.0
Median Arterial flow (mL/min)	144	286	343	242	616	256	260	364	359	377
Median Portal vein flow (mL/min)	871	1103	1283	902	1020	1185	1316	1454	1079	1454
Median liver mass perfusion (mL/g/r)	0.60	0.75	0.76	0.54	1.05	0.81	0.68	0.76	0.77	1.08
Total time (hours) of perfusion	12	24	18	18	24	24	24	12	18	18
Viability achievement	No	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes

**Abbreviations:** Defat- Defatting group; DBD- Donation after brain death; DCD- Donation after circulatory death; ET- Eurotransplant; ALT- Alanine aminotransferase; GGT- Gamma-glutamyltransferase

**7.7.2.3 Table S7.3: Viability criteria achievement by the livers in each group.**

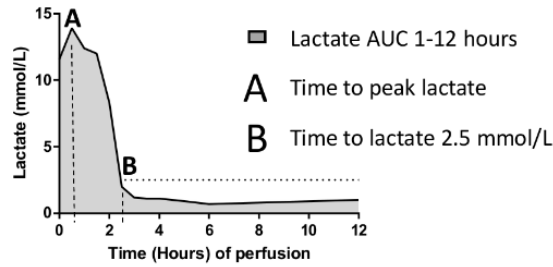
**Table S7.3: Viability criteria achievement by the livers in each group.**

<b>Criteria</b>	<b>Defatting (<i>n</i> = 5)</b>	<b>Control (<i>n</i> = 5)</b>
Lactate clearance ( $\leq 2.5$ mmol/L)	5 (100%)	2 (40%)
pH > 7.3 perfusate	4 (80%)	2 (40%)
Glucose metabolism	5 (100%)	4 (80%)
HA flow (> 150 mL/min)	5 (100%)	4 (80%)
PV flow (> 500 mL/min)	5 (100%)	5 (100%)
Homogeneous perfusion/ soft parenchyma	5 (100%)	5 (100%)
Bile production	5 (100%)	4 (80%)
Viable liver	5 (100%)	2 (40%)

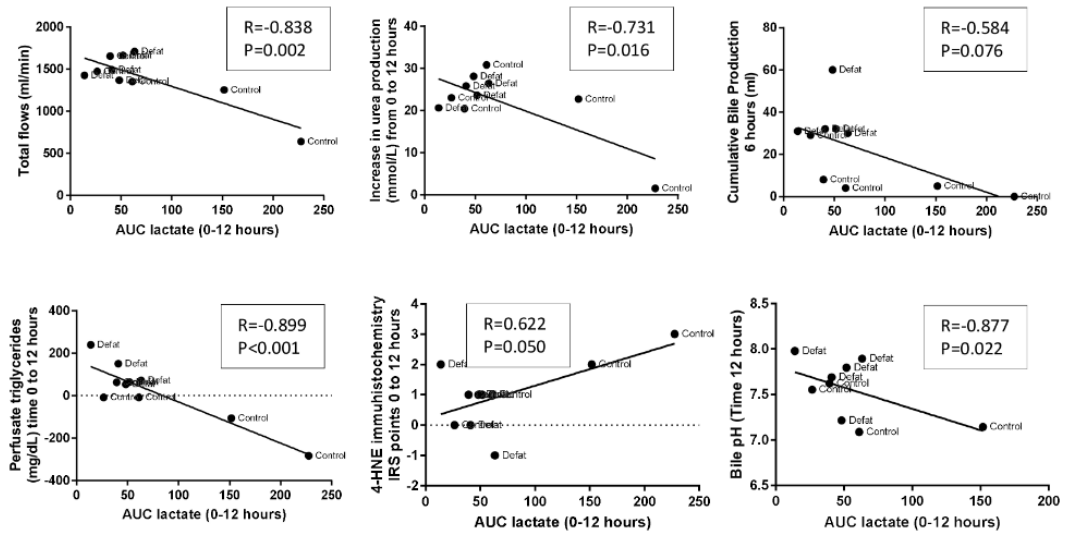
**Abbreviations:** HA- hepatic artery; PV- portal vein.

### 7.7.2.4 Figure S7.1: Lactate clearance dynamics and perfusion parameters in the context of defatting of human livers.

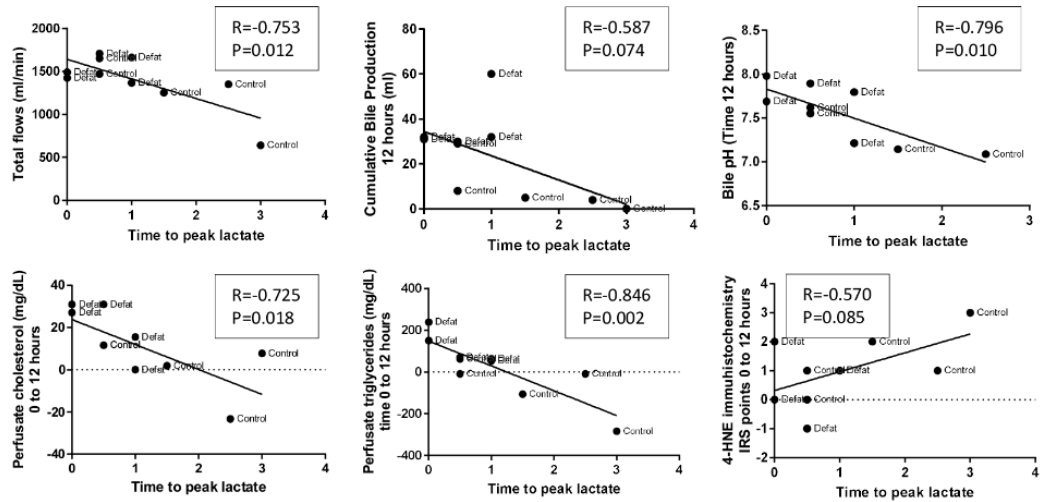
1- Definition of points for analysis of dynamic changes in lactate metabolism.



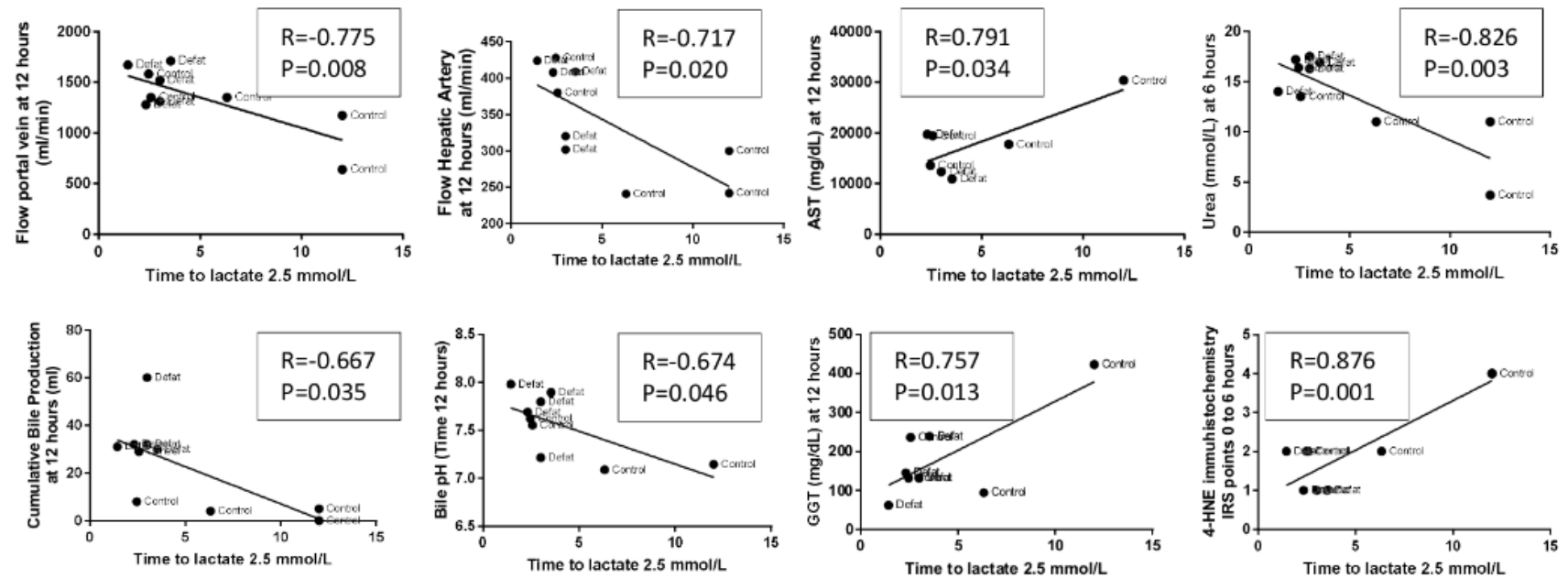
2- Correlation between lactate AUC values and perfusion parameters.



3- Association between time to peak lactate and perfusion parameters



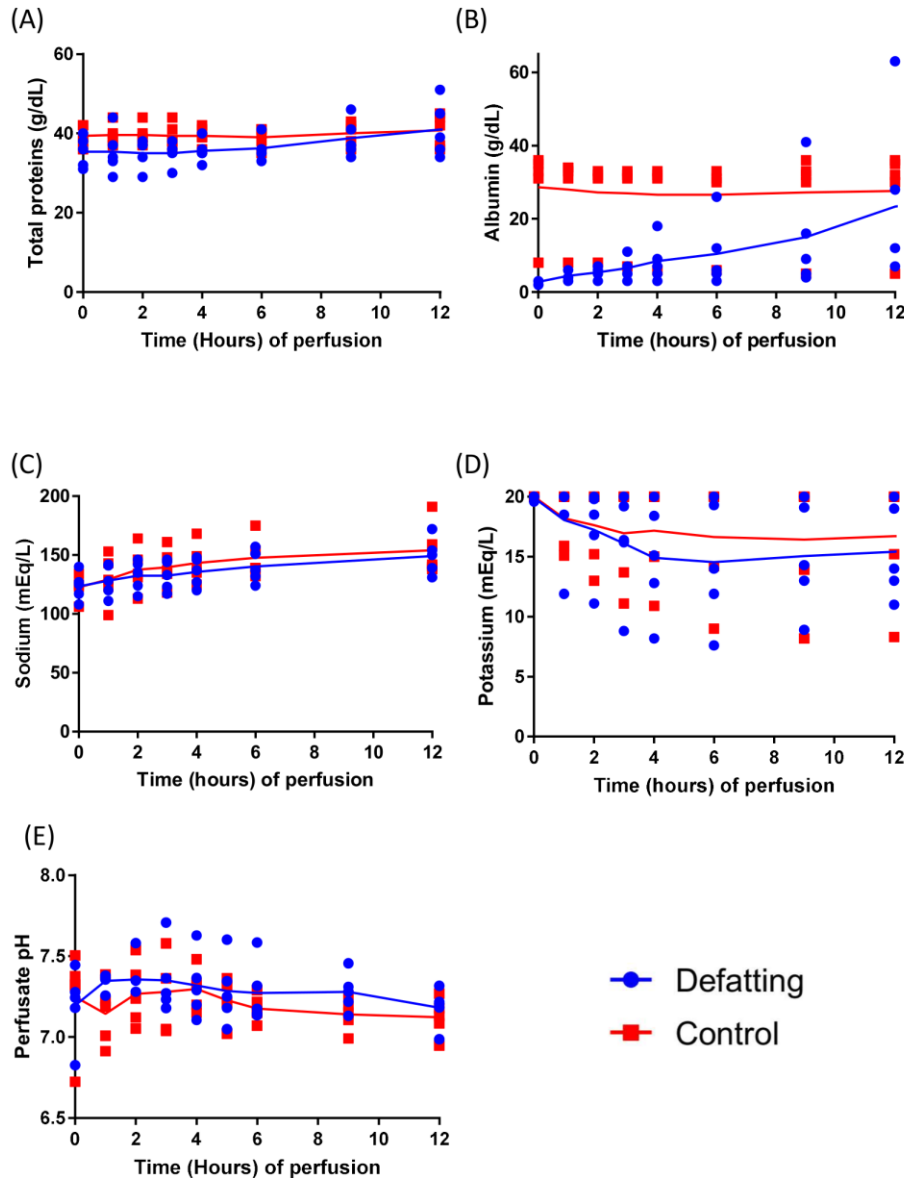
#### 4- Association between time to lactate <2.5 mmol/L and perfusion parameters



**Figure S7.1: Lactate clearance dynamics and perfusion parameters in the context of defatting of human livers.** Series 1: The graph shows the targets analysed for dynamic changes in the lactate metabolism over time. Series 2: for the assessment of dynamic changes in lactate concentration in the perfusate over time the area under the curve (AUC) was determined for each liver. Lactate AUC values from time 0 to 12 hours were compared with other perfusion parameters. There was a strong association between smaller values of lactate AUC with improvements in other metabolic parameters of the organ. Labels identify livers from different groups in the graph. Series 3: the time to achieve the lactate peak (in hours) was correlated with functional parameters and perfusion features. A shorter time to peak of lactate was associated with improvement in diverse parameters of metabolic function of the organ. Series 4: investigation of the correlation between time to lactate less than 2.5 mmol/L (in hours) and perfusion/ metabolic parameters. Data showed that shorter time to reach this target is associated with enhanced metabolic parameters. Pearson's r was used to estimate the relationship between the variables.

### 7.7.2.5. Figure S7.2: Biochemistry analysis of the perfusate.

#### Biochemistry analysis of the perfusate



Panel A: Total proteins (TPROT) were measured in the perfusate and levels were comparable between groups. Panel B: Albumin presented a rising trend in the defatting group and it was stable in the control group. Panel C: Sodium levels measured in the perfusate were comparable between groups. Panel D: Potassium levels decreased quicker within 6 hours of perfusion in the defatting group and for the control levels decreased less and then were flat after initial 2–3 hours. Panel E: Perfusate pH tended to be higher in the control group.

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## Chapter 8

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# IMPACT OF MACHINE PERFUSION OF THE LIVER ON POST-TRANSPLANT BILIARY COMPLICATIONS: A SYSTEMATIC REVIEW

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**A version of this manuscript is published**

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Y.L.B. designed the study, performed the literature review, interpreted the data and wrote the manuscript. A.P.C.S.B. assisted with the literature review and analysis. J.A., L.W. and R.H.B. reviewed critically the manuscript and assisted in editing it. S.A. reviewed critically the manuscript, assisted in the interpretation of the data and editing the manuscript.



## 8.1 Abstract

**AIM:** To review the clinical impact of machine perfusion of the liver on biliary complications post-transplantation, particularly ischaemic-type biliary lesions (ITBL).

**METHODS:** This systematic review was performed in accordance with the Preferred Reporting Systematic Reviews and Meta-Analysis (PRISMA) protocol. The following databases were searched: PubMed, MEDLINE, and Scopus. The keyword ‘liver transplantation’ was used in combination with the free term ‘machine perfusion’. Clinical studies reporting results of transplantation of donor human livers following *ex situ* or *in situ* machine perfusion were analysed. Details relating to donor characteristics, recipients, technique of machine perfusion performed, and postoperative biliary complications (ITBL, bile leak, and anastomotic strictures) were critically analysed.

**RESULTS:** Fifteen articles were considered to fit the criteria for this review. *Ex situ* normothermic machine perfusion was used in six studies, *ex situ* hypothermic machine perfusion in five studies and the other four studies investigated *in situ* normothermic regional perfusion and controlled oxygenated rewarming. While studies on hypothermic machine perfusion and normothermic regional perfusion reported lower rates of ITBL, the interpretation of the results must be performed with caution. This is due to the lack of randomised controlled trials using biliary complications as a primary endpoint and the absence of standardisation for its assessment and reporting. Other biliary complications, such as biliary leak and anastomotic biliary strictures, are reported with similar incidences with all machine perfusion techniques. Restoration of organ to full metabolism during normothermic machine perfusion allows assessment of hepatobiliary function before transplantation, although universally accepted criteria have yet to be validated.

**CONCLUSION:** Machine perfusion of the liver may have potentially a positive impact on post-transplant biliary complications, specifically ITBL, although randomised controlled trials dedicated to assess this issue employing solid and standardised methodology are needed to clarify this question.

## **8.2 Introduction**

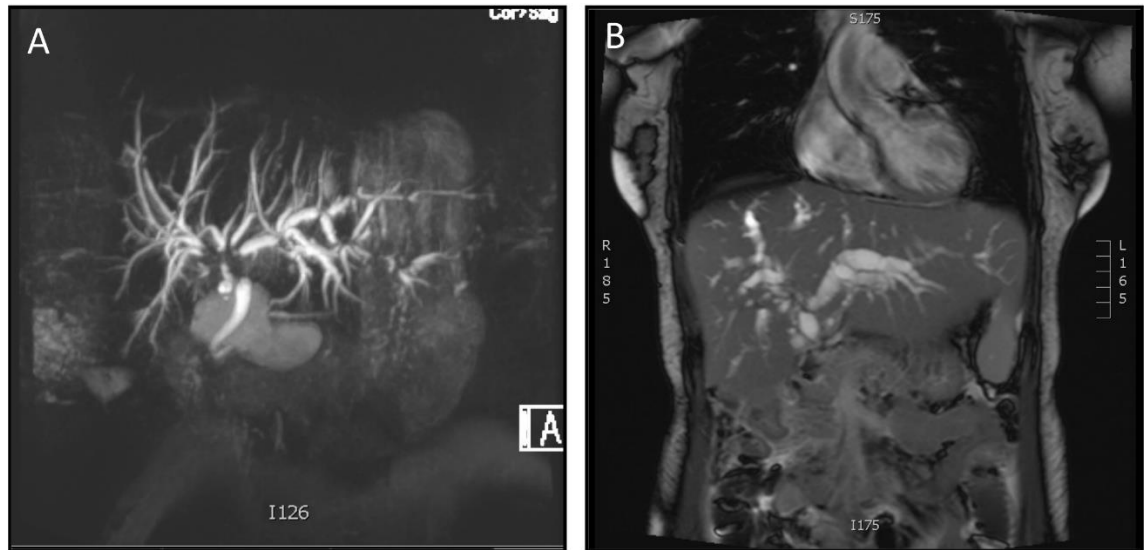
### **8.2.1 Post-transplant biliary complications: the current scenario**

Post-transplant biliary complications often require laborious and costly interventions, placing a heavy burden on health resources and adversely affecting patient outcomes (1, 2). The incidence of these complications is increasing as a result of the growing utilisation of extended criteria donor (ECD) organs, mainly from donation after circulatory death (DCD). Biliary complications such as biliary leak and anastomotic strictures are primarily related to surgical technicalities and are usually successfully managed with endoscopic procedures (3). The most severe form of post-transplant biliary complication is non-anastomotic intrahepatic strictures (NAS). NAS is characterised by the occurrence of diffuse intrahepatic strictures in the biliary tree and it was initially associated with hepatic artery thrombosis (4). The ischaemic donor biliary tree was found to develop necrosis with fibrotic strictures, dilatations and potentially biliary casts (4). Thereafter it was demonstrated that similar lesions occurred in the presence of

a patent hepatic artery without evidence of recurrence of biliary disease. This entity was subsequently classified as ischaemic-type biliary lesion (ITBL) (5).

The reported incidence of ITBL is approximately 10–30% for controlled DCD and 1–3% for donation after brain death (DBD) organs (6–10). Patients generally present with elevated liver function tests suggesting cholestasis (bilirubin, alkaline phosphatase and gamma-glutamyltransferase) within a few months of transplantation and may be asymptomatic initially. Initial work-up includes exclusion of hepatic artery thrombosis and anastomotic biliary strictures. Imaging investigations consist of non-invasive magnetic resonance cholangiopancreatography (MRCP) and computed tomographic cholangiography, or direct cholangiographic methods, such as endoscopic retrograde cholangiopancreatography and percutaneous transhepatic cholangiography. Due to the high reliability of current non-invasive imaging techniques in diagnosing biliary strictures, invasive procedures are currently reserved for scenarios where an intervention is planned, such as stricture dilatation, stenting or stone extraction (11, 12). With ITBL, imaging confirms the presence of fibrotic strictures, in most cases located around the bifurcation of the common bile duct leading to dilatation of the intrahepatic biliary system (1, 8). Figure 8.1 illustrates these typical imaging features of ITBL following liver transplantation. The obstructive strictures cause cholestasis with formation of sludge and casts that predispose to cholangitis, frequently requiring surgical or endoscopic intervention. Despite these measures, approximately 50% of patients with ITBL require re-transplantation or die (13).

**Figure 8.1: Magnetic resonance cholangiopancreatography (MRCP) images of ischaemic-type biliary lesions following liver transplantation.**



The images show two recipients of livers from donation after circulatory death donors that developed ischaemic-type biliary lesions within 60 days following transplantation. Hepatic artery thrombosis and anastomotic biliary strictures were ruled out. A: A typical lesion is seen affecting the bifurcation of the common hepatic bile duct with moderate dilatation of the intrahepatic biliary tree. B: The image shows strictures at the bifurcation of the common hepatic bile duct, diffuse intrahepatic strictures and a severe dilatation of the intrahepatic biliary tree.

Although the pathogenesis of ITBL is still not fully understood a growing body of evidence suggest that it is partially associated with ischaemia-reperfusion injury (IRI) (14, 15). Noack *et al.* in a well-designed *in vitro* study using rat-derived bile duct cells showed that they were more resistant to anoxia than hepatocytes, however during reoxygenation they produced higher amounts of reactive oxygen species (ROS). This was associated with increased rates of bile duct cell death when compared to hepatocytes (16). It has been shown that mitochondrial ischaemic induced injury leads to ROS production during reperfusion which in turn causes oxidative injury and activation of the inflammatory cascade (17, 18). Conversely, clinical series have reported severe injury to the biliary epithelium just after cold static storage (19, 20). Garcia-Valdecasas *et al.* using a porcine transplantation model suggested a direct relationship between prolonged ischaemic times and cell injury (15). Indeed, other clinical series have confirmed the association of longer cold ischaemia time and higher rates of ITBL (21–24). A similar relationship has been observed with warm ischaemia time in DCD liver transplantation (15, 25). A large clinical series of donor bile duct biopsies before liver transplantation showed similar injury to the biliary epithelium after static cold storage (SCS), and that it was exacerbated after reperfusion; however, this did not correlate with the development of ITBL (26). Nevertheless, the authors reported a strong association between ITBL and damage to the peribiliary vascular plexus and peribiliary glands. As progenitor biliary cells are known to reside in the peribiliary glands, the former finding suggests an association between ITBL and an attenuated regenerative capacity of the biliary epithelium (26, 27).

Ischaemic injury is likely to play a major role in ITBL pathogenesis, although other factors have also been shown to be implicated. Immunological mediated injury to the biliary epithelium has been associated with ITBL (28). It may be the result of direct immunological damage to the biliary epithelium via a rejection reaction (29); or, indirect, secondary to the development of

arteriopathy (29, 30). This cross reactivity is described in scenarios of cytomegalovirus infection (30), ABO incompatibility (31) and transplantation for primary sclerosing cholangitis (1). Bile salt toxicity has also been investigated as a potential cause for ITBL by having a direct detergent effect on phospholipid cellular membranes of the biliary epithelium (28). Flushing of the biliary tree during organ procurement is necessary in order to remove all bile salts that could damage cholangiocytes (5, 28). Furthermore, an imbalance in the post-transplant bile composition, with a higher bile salt/ phospholipid ratio, due to inefficient ATP-dependent biliary transporters has been suggested as a predictive factor for ITBL (32). While detail of the pathogenesis of ITBL is beyond the scope of this review, information on the implicated mechanisms can be found in a number of published reviews (9, 28).

### **8.2.2 Machine perfusion of donor livers**

The utilisation of DCD livers is increasing. In 2017, in the United Kingdom, they constituted 28% of the livers transplanted (33). Furthermore, the rising prevalence of donor obesity (body mass index greater than 30 kg/m<sup>2</sup>) and an ageing population continue to compound the risks to those livers (33). These high-risk ECD organs are associated not only with a higher risk of graft dysfunction post-transplantation but also increased rates of ITBL (34). Despite these disadvantages, their utilisation is required to tackle the ever-growing discrepancy between organ donor supply and demand. Machine perfusion (MP) of the liver is being developed as a means of assessment and reconditioning of ECD donors, potentially allowing for safer transplantation of these high-risk livers (34, 35). Different techniques of MP have been developed; it can be performed *in situ* during organ procurement or *ex situ* after the procedure. With regards to livers, the only technique of *in situ* MP described so far is normothermic regional perfusion (8). *Ex situ* MP protocols vary in terms of oxygenation (active or pre-charged oxygenation), perfusate temperature (hypothermic, subnormothermic, gradual rewarming and

normothermic), timing of perfusion (preservation or end-ischaemic) and via of organ perfusion (portal vein alone or dual portal vein and hepatic artery perfusion) (34, 36).

Hypothermic machine perfusion (HMP) has been performed around 10 °C in most studies (37, 38). At this temperature liver metabolism is reduced; and, passive oxygen delivery by diffusion in an oxygen carrier-free perfusate is enough to support the organ (39). The first published clinical series employed pre-charged oxygen delivery to the organs (37), technique that was later followed by active oxygenation of the perfusate (40). Hypothermic oxygenated MP can be performed via portal vein alone (HOPE) or via portal vein and hepatic artery (dual hypothermic oxygenated perfusion – D-HOPE) (41–43). Both techniques have shown the capacity of improve mitochondrial oxidative function prior to rewarming, resulting in increased adenosine triphosphate (ATP) synthesis and a reduction in ROS production, oxidative tissue injury and activation of the inflammatory cascade (42, 43).

Normothermic machine perfusion (NMP) maintains the organ at physiological temperatures (37 °C) and therefore restores full metabolic activity. This enables the possibility of functional or viability assessment prior to transplantation (44, 45). It also opens up a window of opportunity for *ex situ* therapeutic interventions (34). Furthermore, previous studies have reported on the safety of extended normothermic perfusion of organs, which may facilitate transportation and logistical management of busy transplant units (46). However, potential drawbacks of NMP is that it requires obligatorily the inclusion of an oxygen carrier in the perfusate, and NMP inevitably induces reperfusion injury to some extent.

Subnormothermic machine perfusion (SMP) has been performed at around 20 °C in most studies. It encompasses purely SMP and the controlled oxygenated rewarming (COR) from 10 °C to 20 °C (47, 48). The increase in temperature from HMP to SMP is suggested to be enough

to increase liver metabolism to an extent that it would allow assessment of organ function without inducing the detrimental changes associated with organ reperfusion at normothermic temperatures (48). Evidence for the clinical benefits is available for COR perfusions, it was associated with lower markers of hepatocellular injury after transplantation and enhanced graft function through the avoidance of subtle changes in organ temperature (47).

For DCD livers, there are encouraging reports of *in situ* oxygenated normothermic regional perfusion (NRP). It has been successfully applied to controlled DCD donors (withdrawal of life support in patients with irreversible clinical conditions) and uncontrolled DCD (witnessed cardiac arrest without response to resuscitative measures) (8, 49, 50). NRP limits ischaemia and prevents depletion of energy stores prior to SCS and this is suggested to be essential for uncontrolled DCD donors and beneficial for controlled DCD (8).

More recently, combinations of MP techniques have been suggested to merge the advantages of individual protocols, enhancing the rescue of liver function what may potentially improve graft function after transplantation (51, 52). Despite differences between techniques, MP has the potential to limit ischaemic injury to the organ, thus offering a safer preservation environment and an opportunity for organ reconditioning which could mitigate IRI.

As discussed herein, the current evidence shows that cholangiocytes are more vulnerable to IRI than hepatocytes and that the pathogenesis for biliary injury goes beyond IRI. Therefore, investigation of the impact of MP on biliary function specifically, and not only on hepatocellular function, is fundamental. The aim of this review was to investigate the current clinical evidence available regarding the effect of MP on post-transplant biliary complications, focusing on ITBL.

### **8.3 Methods**



This systematic review was performed in accordance with the Preferred Reporting Systematic Reviews and Meta-Analysis (PRISMA) protocol (53).

The following databases were searched for the development of this review: PubMed, MEDLINE and Scopus. The keyword ‘liver transplantation’ was used in combination with the free term ‘machine perfusion’. The literature review was performed until June 20, 2018 and there were no limits on the date for inclusion of publications. The literature search strategy used for one database is presented in the supplementary Table S8.1.

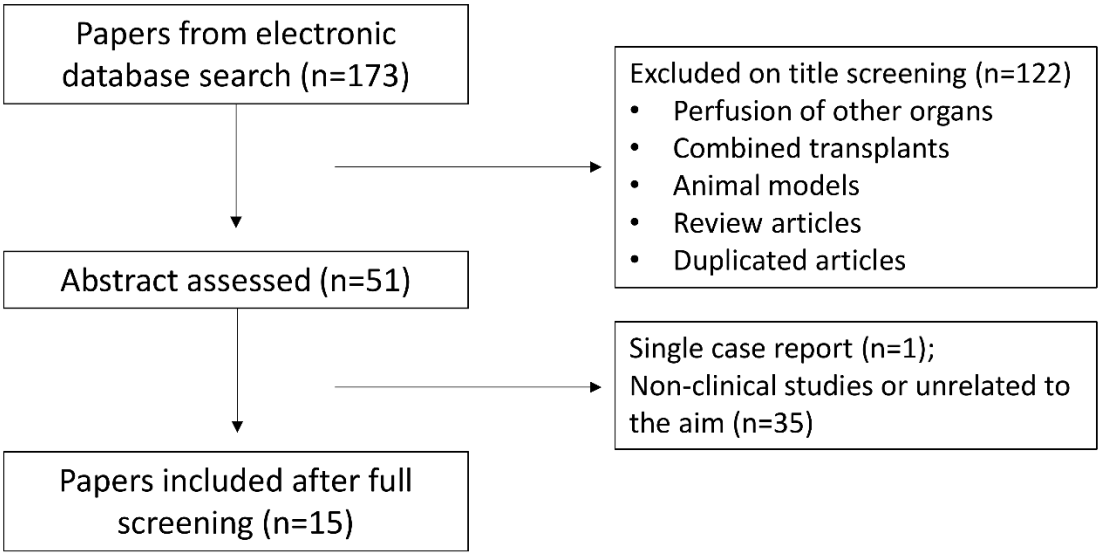
The screening and selection of articles were independently performed by two authors (YLB and APCSB). There was no disagreement in study selection between authors. Manuscript titles that were not related to the main scope of the review were excluded. Full abstracts were then read and excluded if found not to be relevant to the review. Finally, full papers were assessed for eligibility and included in this review. The flow diagram for the literature selection process is shown in Figure 8.2.

Inclusion criteria were: (1) clinical studies reporting results of transplantation of donor human livers following *ex situ* or *in situ* MP; (2) articles written in English and published. Exclusion criteria were: (1) absence of transplantation following MP; (2) exclusively animal models; (3) single case report; (4) review articles; and, (5) articles not written in English.

Details relating to donor characteristics (type, age, donor risk index [DRI], warm ischaemic time [WIT], cold ischaemia time [CIT]), recipients (age, model for end-stage liver disease [MELD]), perfusion (type of perfusion, oxygenation, timings) and postoperative biliary complications (ITBL, leak and anastomotic strictures) were retrieved from each manuscript and critically analysed. Studies were assessed in terms of study design, methods and outcomes. No

review protocol was registered before this review was started. No simplifications or assumptions were made, and any identified risk of bias is discussed throughout the review.

**Figure 8.2: Study flow diagram for systematic review of the literature on the impact of machine perfusion of the liver and post-transplant biliary complications.**



Following literature search duplicate articles were excluded and the titles screened. The selected abstracts were then read and non-clinical studies or reports unrelated to the aim of the review were excluded.

## **8.4 Results**

Fifteen articles were considered to fit the criteria for this review. A diagrammatic summary of the screening process is provided in Figure 8.2.

### **8.4.1 Machine perfusion and ischaemic-type biliary lesions (ITBL)**

Eight out of fifteen clinical studies utilised an end-ischaemic model of MP (MP commenced after a variable period of SCS), four studies utilised preservation MP (MP from organ procurement up to transplantation) and three employed NRP. NMP was used in six studies, HMP in five studies and the other four studies investigated NRP and COR. HMP with active perfusate oxygenation (HOPE and D-HOPE) studies were seen to be currently focused on DCD organs and HMP with pre-charged oxygenation on DBD organs. NMP studies used both donor types, however preservation studies explored a higher proportion of DBD compared to DCD organs. The contrary was seen for end-ischaemic NMP.

Donor and recipient characteristics, of the cases included in individual studies, are presented in Table 8.1. It also reports the rates of ITBL. Table 8.2 describes the incidence of bile leak and anastomotic biliary stricture within the different studies. Studies characteristics were described therein, as it was their design.

**Table 8.1: Comparison between donor, recipient, perfusion characteristics and the reported rates of ischaemic-type biliary lesions.**

Author	Year	Perf. type	Timing MP	N	Donor age	Donor risk index	Recipient age	Recipient MELD	DBD (n)	DCD (n)	DBD ITBL	DCD ITBL	CIT (minutes)	Func. WIT (minutes)	Re-Tx (n)
<b>Ex situ Normothermic Machine Perfusion</b>															
Nasralla <i>et al.</i> (46)	2018	NMP	Preserv.	121	56 (16–84)	1.7 <sup>€</sup>	55	13 (6–35)	87	34	7.4%	11.1%	126	21	3
Selzner <i>et al.</i> (54)	2016	NMP	Preserv..	10	48 (17–75)	1.9	57	21 (8–40)	8	2	0%	0%	103	NA	0
Bral <i>et al.</i> (56)	2017	NMP	Preserv.	9	56 (14–71)	1.6 (0.9–2.7)	53 (28–67)	13 (9–32)	6	3	0%	0%	167 (95–293)	22	0
Ravikumar <i>et al.</i> (55)	2016	NMP	Preserv.	20	58 (21–85)	NA	NA	12 (7–27)	16	4	0%	0%	NA	21	0
Watson <i>et al.</i> (58)	2018	NMP	End-Isc.	22	57	2.3	NA	NA	6	16	0%	25.0%	386	12	3
Mergental <i>et al.</i> (57)	2016	NMP	End-Isc.	5	49 (29–54)	2.3	56 (47–66)	8 (8–13)	1	4	0%	0%	422	28	0
<b>Ex situ Hypothermic Non-Oxygenated Machine Perfusion</b>															
Guarrera <i>et al.</i> (59)	2015	HMP	End-Isc.	31	57 (+/– 18)*	1.9 (±0.5)*	57 (±8.0)*	19 (±5.9)*	31	0	9.7%	NA	558	NA	0
Guarrera <i>et al.</i> (37)	2010	HMP	End-Isc.	20	39 (+/– 2.5)*	NA	55 (±6.2)*	17 (±7.4)*	20	0	5.0%	NA	306	26	0
<b>Ex situ Hypothermic Oxygenated Machine Perfusion</b>															
van Rijn <i>et al.</i> (43)	2017	DHOP E	End-Isc.	10	53 (47–57)	1.9 (1.5–2.2)	57 (54–62)	16 (15–22)	0	10	NA	10.0%	331	15	0
Dutkowski <i>et al.</i> (38)	2015	HOPE	End-Isc.	25	54 (36–63)	NA	60 (57–64)	13 (9–15)	0	25	NA	0%	188 (141–264)	31 (26–36)	0
Dutkowski <i>et al.</i> (40)	2014	HOPE	End-Isc.	8	54 (NA)	2.2 (NA)	60 (NA)	12 (NA)	0	8	NA	0%	141 (NA)	31 (22–41)	0
<b>In situ Normothermic Regional Perfusion</b>															
De Carlis <i>et al.</i> (60) <sup>¥</sup>	2017	NRP	NRP	7	48*	NA	54*	10.6*	0	7	NA	0%	414*	33	0
Oniscu <i>et al.</i> (49)	2014	NRP	NRP	11	46 (16–74)	NA	68 (43–74)	NA	0	11	NA	0%	389 (169–450)	26 (13–48)	0
Minambres <i>et al.</i> (50)	2017	NRP	NRP	11	58 (50–67)	NA	55 (±13)*	NA	0	11	NA	0%	266 (±82.7)*	12 (11–16)	0
<b>Controlled Oxygenated Rewarming</b>															
Hoyer <i>et al.</i> (47)	2016	COR	End-Isc.	6	58 (51–71)	1.9 (1.5–2.5)	52 (43–65)	18 (11–23)	6	0	0%	NA	508 (369–870)	NA	0

\*Data presented as median or median (±Standard deviation), if available. Otherwise, all data presented as median (Interquartile range). ¥Combined hypothermic oxygenated machine perfusion after normothermic regional perfusion. Six uncontrolled DCD were included in this study. € Eurotransplant DRI. Abbreviations: MP- Machine perfusion; MELD- Model for end stage liver disease; DBD- Donation after brain death; DCD- Donation after circulatory death; ITBL- Ischaemic-type biliary lesions; CIT- Cold ischaemic time; Func. WIT- Functional warm ischaemic time; Re-Tx- Re-transplantation; NA- Not applicable or not available; Preserv.- Preservation; End-Isc.- End ischaemic; NMP- Normothermic machine perfusion; HMP- Hypothermic machine perfusion; DHOPE- Dual vessel hypothermic oxygenated machine perfusion; HOPE- Hypothermic oxygenated machine perfusion; NRP- Normothermic regional perfusion; COR- Controlled oxygenated rewarming.

**Table 8.2: Prevalence of bile leak and anastomotic biliary strictures between clinical studies using different techniques of machine perfusion of donor livers.**

Author	Year	Study design	Perfusion type	Timing Machine Perfusion	N	DBD (n)	DCD (n)	Bile leak (n)	Anastomotic stricture (n)
<b><i>Ex situ</i> Normothermic Machine Perfusion</b>									
Nasralla <i>et al.</i> (46)	2018	RCT	NMP	Preservation	121	87	34	0	0
Selzner <i>et al.</i> (54)	2016	PS	NMP	Preservation	10	8	2	0	0
Bral <i>et al.</i> (56)	2017	PS	NMP	Preservation	9	6	3	0	0
Ravikumar <i>et al.</i> (55)	2016	PS	NMP	Preservation	20	16	4	0	4 (DBD)
Watson <i>et al.</i> (58)	2018	DS	NMP	End-Ischaemic	22	6	16	0	0
Mergental <i>et al.</i> (57)	2016	DS	NMP	End-Ischaemic	5	1	4	0	0
<b><i>Ex situ</i> Hypothermic Non-Oxygenated Machine Perfusion</b>									
Guarrera <i>et al.</i> (59)	2015	PS	HMP	End-Ischaemic	31	31	0	1	0
Guarrera <i>et al.</i> (37)	2010	NCS	HMP	End-Ischaemic	20	20	0	1	1
<b><i>Ex situ</i> Hypothermic Oxygenated Machine Perfusion</b>									
van Rijn <i>et al.</i> (43)	2017	PS	DHOPE	End-Ischaemic	10	0	10	0	2
Dutkowski <i>et al.</i> (38)	2015	PS	HOPE	End-Ischaemic	25	0	25	5 (in total)	
Dutkowski <i>et al.</i> (40)	2014	PS	HOPE	End-Ischaemic	8	0	8	1	1
<b><i>In situ</i> Normothermic Regional Perfusion</b>									
De Carlis <i>et al.</i> (60) <sup>‡</sup>	2017	DS	NRP	NRP	7	0	7*	0	1
Oniscu <i>et al.</i> (49)	2014	DS	NRP	NRP	11	0	11	1	1
Minambres <i>et al.</i> (50)	2017	DS	NRP	NRP	11	0	11	NA	NA
<b>Controlled Oxygenated Rewarming</b>									
Hoyer <i>et al.</i> (47)	2016	PS	COR	End-Ischaemic	6	6	0	NA	NA

<sup>‡</sup>Combined hypothermic oxygenated machine perfusion after normothermic regional perfusion. Six uncontrolled DCD were included in this study. Abbreviations: RCT- Randomised controlled trial; PS- Single-arm non-randomised pilot study; DS- Descriptive study; NCS- Non-randomised cohort studies; DBD- Donation after brain death; DCD- Donation after circulatory death; NA- Not applicable or not available; NMP- Normothermic machine perfusion; HMP- Hypothermic machine perfusion; DHOPE- Dual vessel hypothermic oxygenated machine perfusion; HOPE- Hypothermic oxygenated machine perfusion; NRP- Normothermic regional perfusion; COR- Controlled oxygenated rewarming.

#### 8.4.2 Normothermic machine perfusion and post-transplant biliary complications

The largest randomised controlled clinical trial involving NMP as a preservation strategy was recently published by Nasralla *et al.* (46). Following procurement, transplantable livers were randomised and allocated to the intervention group that had NMP up to the point of transplantation or a control group that had conventional SCS. From the 121 livers perfused, 87 were from DBD donors and 34 from DCD donors. Results did not show statistically significant differences in bile duct complications between groups, with one patient in each arm developing ITBL within the first year, both requiring re-transplantation. On MRCP, the rate of NAS was not significantly different between groups for DBD (NMP 7.4% vs. SCS 5.4%;  $p = 0.678$ ) and DCD (NMP 11.1% vs. SCS 26.3%;  $p = 0.180$ ). The incidence of anastomotic strictures was also similar for DBD or DCD organs (NMP 40.7% vs. SCS 41.8%;  $p = 0.909$ ; and, NMP 48.1% vs. SCS 57.9%;  $p = 0.515$ , respectively) (46).

Other clinical studies investigating NMP using a preservation approach (54–56) involved smaller patient numbers, the majority of which were from DBD donors, and did not specifically report the incidence of ITBL (Table 8.1). Ravikumar *et al.* published the first phase 1 clinical trial demonstrating the safety and feasibility of NMP in a preservation approach, as an alternative to SCS (55). In all, 20 donor livers (16 DBD and 4 DCD) were transplanted following NMP. The 30-day graft survival was similar to static cold stored livers and the median peak aspartate aminotransferase within the first 7 postoperative days was lower. In terms of biliary complications, the authors reported the occurrence of four cases of anastomotic biliary strictures in the NMP group (55).

The two studies of NMP after a period of SCS (end-ischaemic model) involved organs that were deemed too high risk for transplantation (57, 58). These studies predominantly used DCD

livers and applied predefined viability criteria prior to transplantation. Mergental *et al.* did not observe any biliary complications at 7 months of follow up post-transplantation (57). Watson *et al.* reported the occurrence of 4 cases of ITBL in 16 DCD liver transplants, of which 3 needed re-transplantation (58). The authors of the latter study suggested that NMP per se does not prevent ITBL but may provide biomarkers to identify livers that are high risk, such as maximum bile pH > 7.5 and bile glucose  $\leq 3$  mmol/L or  $\geq 10$  mmol less than perfusate glucose (58).

#### **8.4.3 Hypothermic machine perfusion and post-transplant biliary complications**

The first clinical study using HMP prior to transplantation was performed by Guarrera *et al.* (37). Twenty DBD livers were perfused after a period of SCS in a non-actively oxygenated model of HMP. ITBL rate was reported as 5%, half of the incidence of the control matched cohort that was subjected to SCS. In addition, there was one case of bile leak and one report of anastomotic biliary stricture (37). The same approach was repeated later in a study of DBD livers declined by the United Network for Organ Sharing region for transplantation (59). The authors found a significant decrease in the rate of biliary stricture in comparison with SCS (10% vs. 33%,  $p = 0.031$ ). One report of bile leak was noted in the HMP group and three in SCS respectively (Table 8.2).

Following these initial studies, the Zurich group developed the concept of HOPE, with active oxygenation of the perfusate, and applied this MP strategy to DCD donors (38, 40). Their first clinical trial was published in 2015, reporting the results of transplantation of 25 DCD livers (38). The authors reported no cases of ITBL at 1-year follow-up of patients who received perfused DCD livers, whereas control livers subjected to SCS developed a significantly higher rate of ITBL (0/25 vs. 11/50,  $p = 0.013$ ). The same benefit of HOPE was not seen for

extrahepatic biliary complications, as the reported rates of leaks and anastomotic strictures were similar (HOPE 5/25 vs. Control 12/50) (38).

The Groningen group published the first clinical series using D-HOPE in 2017 (43). Ten DCD livers were transplanted following 2 hours of D-HOPE, one patient in the perfusion group developed ITBL compared to 7 out of 20 in the control group. The case in the D-HOPE group was described as NAS in segments II and III of the liver and was managed with endoscopic stenting. Three control livers which developed ITBL required re-transplantation. The rate of anastomotic biliary strictures was comparable between groups (D-HOPE 2 vs. Control 3,  $p = 1.000$ ) as was the reported rate of biliary cast formation (D-HOPE 3 vs. Control 3,  $p = 0.372$ ) (43).

#### **8.4.4 Normothermic regional perfusion and post-transplant biliary complications**

The first series reporting the results for transplantation of livers following NRP was published in 2014 by Oniscu *et al.* (49) The authors reported the results of transplantation of 11 controlled DCD livers, with a minimum follow-up of three months, with no clinical or radiological evidence of ITBL. One patient developed an anastomotic stricture, treated endoscopically by cholangio-pancreatography (exact intervention performed is not described), and one patient had a bile leak (49). Minambres *et al.* 2017, studying controlled DCD transplantation after NRP, reported no cases of ITBL after 1-year follow-up (50). De Carlis *et al.* 2017 performed NRP on one controlled DCD liver and six uncontrolled DCD (60). On arrival at the transplant centre, the livers were subjected to D-HOPE until transplantation. No cases of ITBL were observed and one patient had an anastomotic biliary stricture 45 days after transplantation, which was successfully treated with endoscopic stenting (60).



In terms of SMP, Hoyer *et al.* reported transplantation of six DBD livers following COR perfusion (47). No biliary complications were reported within a follow-up period of six months.

## **8.5 Discussion**

Post-transplant biliary complications are associated with high rates of morbidity and re-transplantation and are a major obstacle to the wider clinical utilisation of ECD livers. Although there is a growing body of evidence suggesting that MP can offer safer organ preservation when compared to SCS, and also offer an opportunity for organ assessment and/ or reconditioning prior to transplantation, properly designed randomised controlled trials using biliary complications as a primary endpoint are still missing (38, 43, 46, 49, 58). In this review, the available literature investigating the impact of MP on post-transplant biliary complications, with special reference to ITBL has been assessed. MP techniques suggested to have the potential to alleviate IRI, such as HMP and NRP, have also reported lower rates of ITBL, however, these results should be considered in face of the limitations of study designs, restricted sample size of pilot studies and inconsistencies in the definition of ITBL. Other biliary complications, such as biliary leak and anastomotic biliary strictures, are reported with similar incidences with MP techniques.

Liver IRI is thought to be a major driver of biliary injury and, therefore, it is associated with complications following transplantation. More specifically, during ischaemia, without oxygen as a terminal acceptor of electrons in the electron transport chain, succinate accumulates and acts as a store for electrons. Succinate oxidation during the early stage of reperfusion, blocks mitochondrial complex II of the electron transport chain resulting in a reverse flow of electrons towards mitochondrial complex I leading to accentuated leakage of electrons, and generation of ROS (61). Various experimental findings using the HOPE technique have shown that oxygen

at hypothermic temperatures is able to promote mitochondrial metabolism of succinate prior to reperfusion (36, 42, 62). By re-establishing adequate mitochondrial oxidative function, HOPE is able to recover ATP stores, since during hypothermia mitochondria have lower energy requirements due to a minimum activation of the organ metabolism. Therefore, mechanistically, HOPE can in theory prevent the reverse flow of electrons during reperfusion, ROS generation and activation of the inflammatory cascade (36). These factors may mitigate IRI, which would be beneficial not only for hepatocellular function but also for the prevention of further biliary injury.

Extensive research focussing on the effect of oxygenated HMP on post-transplant biliary complications has been performed by the Groningen group. In a recent publication exploring the effects of D-HOPE on bile duct biopsies from a previous published series of cases, they showed less injury to deep and periluminal peribiliary glands after reperfusion during transplantation in the perfused group in comparison with SCS control livers (43, 63). Peribiliary glands have been described as stores for biliary progenitor cells, therefore injury to them would potentially decrease the regenerative capacity of the biliary system (64, 65). The authors acknowledge that definitive evidence to support this would require a clinical randomized trial that has since been initiated at their centre (63).

Preservation NMP shortens ischaemic injury and offers a more physiological environment for the organ before transplantation. Nevertheless, as previously discussed, the injury to biliary cells might not be restricted to an ischaemic mechanism but may also be worsened during reperfusion. NMP restores the full metabolism of the organ, inevitably resulting in the production and circulation of ROS and potential activation of the inflammatory response leading to tissue injury (66). On the other hand, restoration of the organ to full metabolism allows assessment of hepatobiliary function before transplantation, although, universally

accepted criteria have yet to be validated (35). Watson *et al.* suggested bile pH and glucose content as markers of bile duct injury and associated those with the development of ITBL (58).

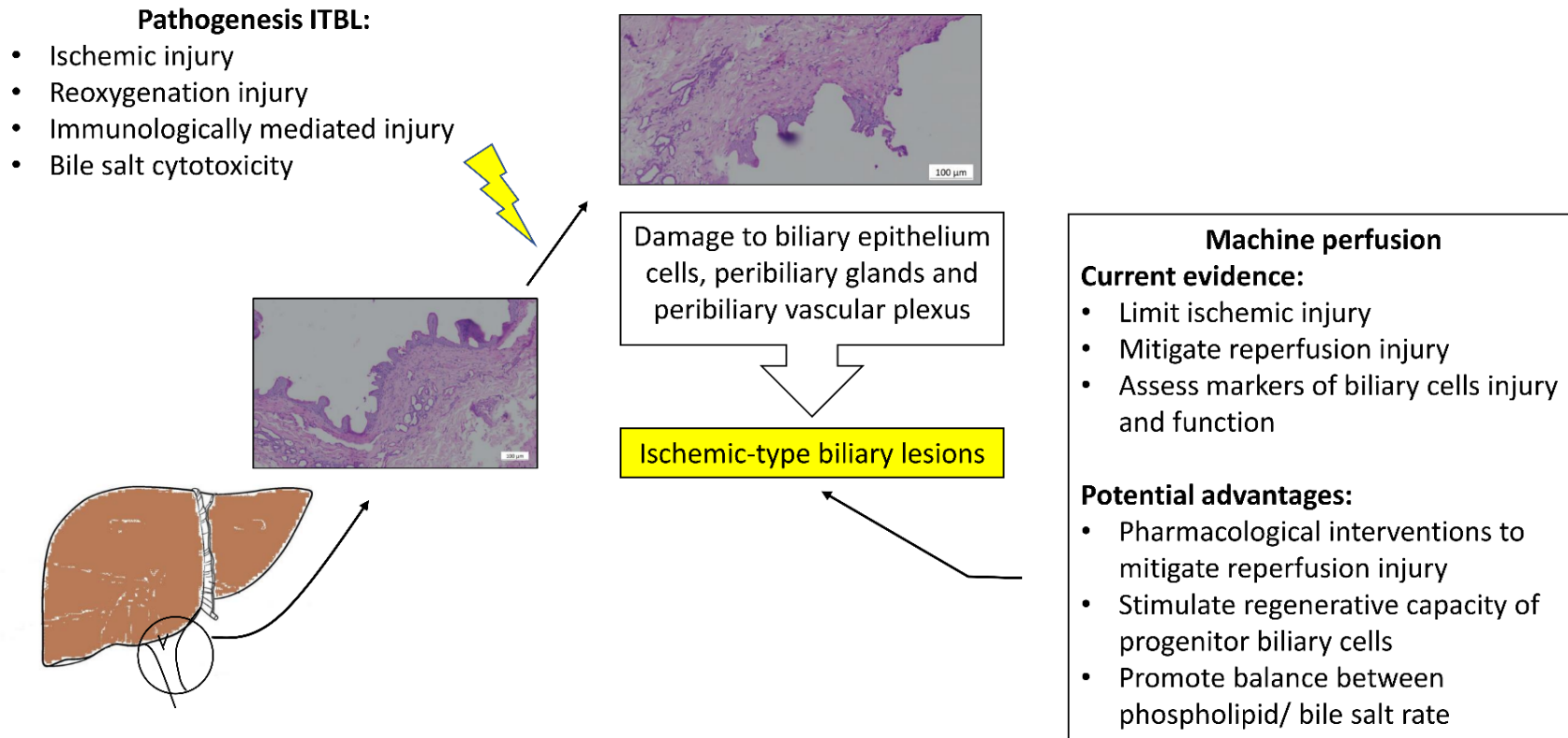
Promisingly, *in situ* NRP has shown excellent biliary outcomes after transplantation of DCD livers (49, 50, 60). NRP may potentially prevent ischaemic injury and deterioration of ATP stores during organ procurement. In addition, NRP allows assessment of the liver metabolism even before SCS (8). Despite these points, there is no mechanistic evidence available to demonstrate any alleviation in IRI after reperfusion.

The present body of work has several limitations; thus, interpretation of the results must be performed with caution. First, thus far, there is no randomised controlled trial using biliary complications post-transplantation as a primary endpoint. Few clinical studies from each MP technique are available and most are pilot studies with small sample size, which limits definitive interpretation of the data. Second, there is lack of standardisation in the methods to assess these complications or to define ITBL radiologically. For example, MRCP was not performed in all studies evaluated. Moreover, MRCP is not routinely requested for patients after transplantation unless clinically indicated. In addition, there is no widely accepted criteria to report the findings on the MRCP, findings in which the significance without clinical correlation is not clear. Furthermore, donor livers and recipient characteristics, as well as machine perfusion technique protocols, exhibit a high degree of variability between studies. Thus far, there has been no standardisation in terms of methodology and reporting of results. Some studies further neglect to report important data variables, such as donor risk index, recipient age, and recipient MELD and CIT. All these features are presented in Table 8.1 to allow an unbiased assessment of the retrieved information by the reader. All together, these factors require attention before making comparisons between various modalities of MP with regards to biliary complications post-transplantation.

Up to now, most of clinical series studying MP have focussed on the evaluation of hepatocellular function rather than biliary function and injury. Despite the subject of this review being a relevant topic with important clinical implications, the direct effects of MP on biliary tree integrity are still relatively under-researched. More clinical randomised trials must be reported in the field over the next few years.

Higher rates of ITBL following transplantation of ECD livers, mainly DCD, place a major restraint on the wider use of these marginal livers. Each technique of MP may offer different advantages and they all have the potential to tackle this problem. A preclinical study has shown that a combination of HOPE and NMP increased the rescue of metabolic parameters of high-risk ECD organs (52). This approach may derive benefits from the individual methods, thus possibly optimising gains also in terms of biliary function. Pharmacological interventions during NMP may potentially alleviate IRI, positively affecting biliary cells (67), and may have a direct effect on post-transplant biliary complications. Supplementation of the perfusate with substances that may induce proliferation and maturation of progenitor cells from peribiliary glands may be a feasible option to be considered (9). We hypothesize that therapies promoting increase in secretion of phospholipids and cholesterol in the bile would equilibrate the phospholipids/ bile salts balance mitigating further injury to the biliary tree. Although promising, these are options that still need to be explored in future studies. A diagrammatic summary of the current and future impact of MP on ITBL is presented in Figure 8.3.

**Figure 8.3: Diagrammatic summary of the current evidence for the impact of machine perfusion of the liver on post-transplant ischaemic-type biliary lesions and future perspectives.**



The current evidence suggests that ischaemic-type biliary lesions have a multifactorial pathogenesis. These diverse factors lead to injury to the biliary epithelium, peribiliary glands and peribiliary vascular plexus. Currently, there is evidence proposing potential benefits of machine perfusion on post-transplant ischaemic-type biliary lesions. The figure summarises those and possible future interventions that could enhance increase these benefits further.

## **8.6 Conclusion**

The high incidence of post-transplant biliary complications, specifically ITBL, is a major constraint to wider utilisation of ECD livers. While MP is currently considered a promising tool to increase ECD utilisation, definitive clinical evidence for a positive impact of MP on post-transplant biliary complications is still pending. Despite some clinical series suggesting a potentially beneficial effect of MP on ITBL rates, randomised controlled trials powered for its occurrence as a primary endpoint are needed to confirm this data. In addition, further efforts should be taken by the scientific community to promote standardisation in the assessment and definition of ITBL between studies, including the use of similar imaging protocols and reporting systems.

## 8.7 Appendices

### 8.7.1 Table S8.1: Literature search strategy example

Database	Strategy
PubMed	'liver transplantation' (MeSH) AND 'machine perfusion' (text)

**Abbreviations:** MeSH: Medical subject heading; text: Text word

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## **Chapter 9**

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# **GENERAL DISCUSSION AND CONCLUSION: LIMITATIONS AND FUTURE PERSPECTIVES OF THE THESIS**

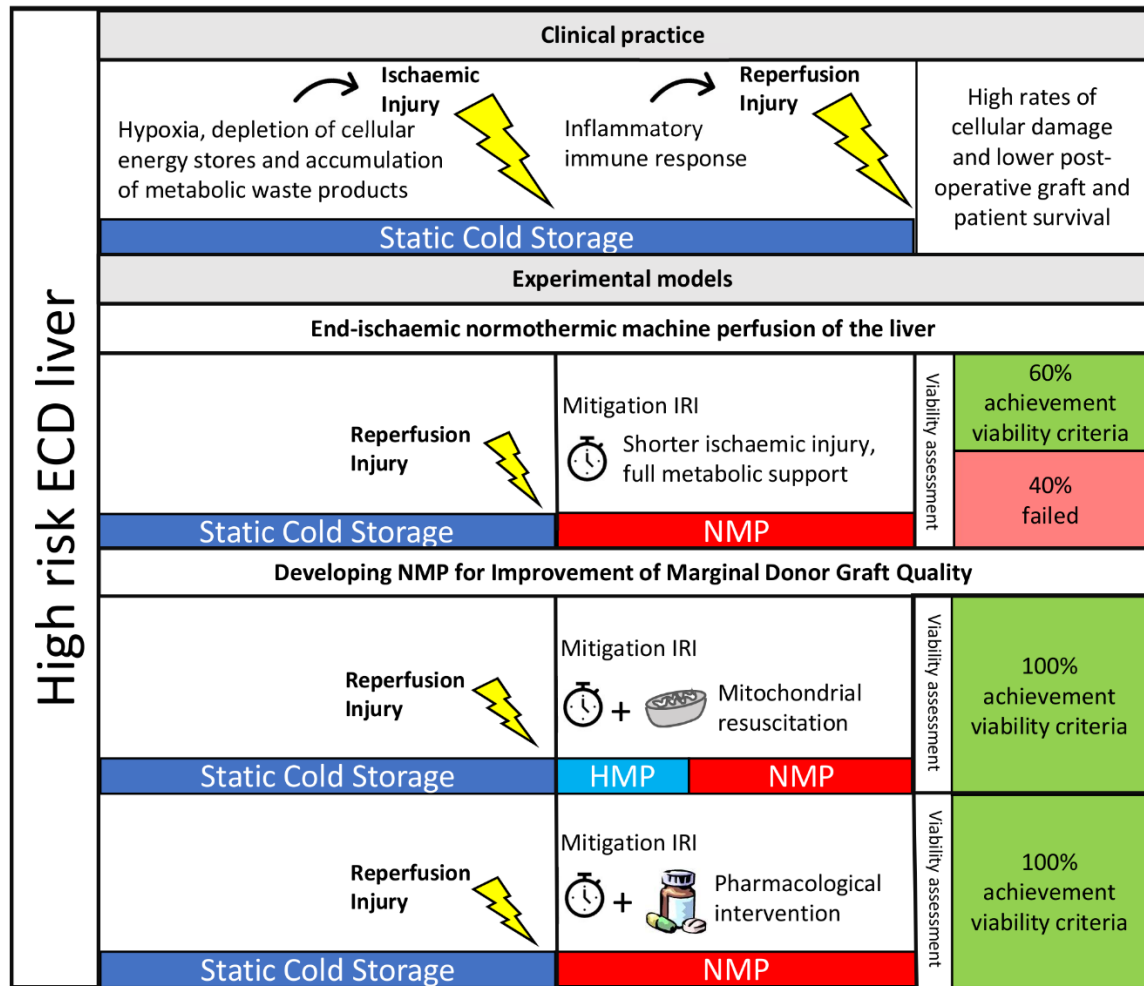
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This thesis examined how to develop *ex situ* normothermic machine perfusion of the liver to improve the reconditioning of high-risk extended criteria donor organs. Two possible alternatives were investigated (Figure 9.1): (1) The use of hypothermic oxygenated machine perfusion as a therapeutic intervention preceding normothermic machine perfusion; and (2) the delivery of a pharmacological combination of drugs targeting the hepatic lipid metabolism during normothermic perfusion to enhance the metabolic support to donor organs.

**In detail, this thesis studied:**

- **The feasibility of developing a unified protocol of machine perfusion combining hypothermic and normothermic machine perfusions; and whether the combined protocol would derive specific benefits of the individual techniques.**
- **The feasibility of using a single acellular haemoglobin-based oxygen carrier perfusate for an uninterrupted combined protocol of hypothermic and normothermic machine perfusions, the Cold-to-Warm machine perfusion of the liver.**
- **The cytotoxicity *in vitro* of a combination of drugs targeting the enhancement of hepatic lipid metabolism towards primary human cells of the liver: hepatocytes, cholangiocytes and intrahepatic endothelial cells. In addition, the effectiveness of the drugs in decreasing the intracellular lipid content of primary human hepatocytes loaded with fat was examined.**
- **The feasibility of using normothermic machine perfusion as a tool for the delivery of a pharmacological intervention targeting the hepatic lipid metabolism of human livers; and the effectiveness of this treatment in enhancing the hepatic lipid metabolism of a whole human organ, decreasing the intracellular lipid content and improving the metabolic support to other cellular processes.**

**Figure 9.1: Diagrammatic summary of the strategies examined in this thesis for developing normothermic machine perfusion of the liver for improvement of marginal donor graft quality.**



High-risk extended criteria donor (ECD) livers are more vulnerable to the cellular damage caused by ischaemia followed by the insult of warm blood during the revascularisation. The significant cellular damage impairs the metabolic function of the organs and thus leads to a high risk of delayed graft function or even graft loss postoperatively. End-ischaemic normothermic machine perfusion (NMP) alleviates the damage associated with ischaemia-reperfusion injury (IRI) because it limits the period of cold ischaemia and delivers nutrients and oxygen to support the full metabolism of the organs. Whilst limitation of the ischaemic insult is beneficial, it does not prevent the amplification of the inflammatory immune response associated with reperfusion and, therefore, approximately 40% of the high-risk ECD livers fail to recover metabolic function sufficiently to be deemed transplantable. In Chapters 3 and 4 the use of hypothermic oxygenated machine perfusion (HMP) as a therapeutic intervention targeting resuscitation of mitochondrial bioenergetic and oxidative status prior to NMP, in a combined protocol, was explored. This combination successfully enhanced the return of metabolic functions to parameters considered adequate for transplantation. Alternatively, the delivery of a pharmacological intervention aiming to increase the hepatic lipid metabolism was investigated. The boosted lipid metabolism enhanced the metabolic support to other cellular and mitochondrial processes. This effect also mitigated oxidative tissue damage and the propagation of the inflammatory response with lower tissue injury and full recovery of metabolic function of the organs.

## 9.1 The development of the Cold-to-Warm protocol of machine perfusion of the liver

Clinical evidence of benefits of hypothermic and normothermic machine perfusion (MP) of donor livers individually was reviewed in **Chapters 1** and **2**. The proposition and stepwise development of the Cold-to-Warm protocol of *ex situ* MP was presented in **Chapters 3** and **4**. The combined protocol of hypothermic and normothermic MP, in the two studies performed, was shown to optimise mitochondrial oxidative function replenishing cellular adenosine triphosphate (ATP) stores during the hypothermic phase; and to mitigate the expression of markers of oxidative tissue damage, reactive oxygen species' production and activation of inflammatory cells. Ultimately, organs submitted to the combined protocol were more likely to recover sufficient metabolic function to be considered transplantable. Therefore, considering that the ultimate goal of MP technology is to increase reconditioning and utilisation of high-risk extended criteria donor (ECD) organs, the combination of hypothermic and normothermic perfusions needs to be considered as a plausible alternative for future clinical studies.

Although this combined perfusion protocol seems promising, it has some limitations. Whilst the use of human donor organs for research eliminates issues with animal studies, imposed by the inherent variability in response between species, their scarcity limits sample size and the intrinsic heterogeneity compromises an exact match between experimental groups. Nevertheless, admittedly, animal studies offer some other advantages, such as the possibility of large numbers of animals which can be studied, superior comparability between experimental groups and easier implementation of interventional studies. Therefore, whilst missing, data from animals may potentially provide important information on the benefits of the proposed strategy. The developed combined protocol of hypothermic and normothermic MP is suggested to enhance the reconditioning of high-risk ECD livers because they more frequently reached



viability criteria previously used for clinical transplantation; however, final viability assessment for organ function would be transplantation, not performed in these preclinical studies (1).

## **9.2 Future research questions—The Cold-to-Warm liver machine perfusion protocol**

After the development of the Cold-to-Warm MP protocol in **Chapters 3 and 4**, there are key topics to be possibly investigated in future studies.

### **9.2.1 The development of a clinical protocol of Cold-to-Warm MP of the liver**

The results from the preclinical studies involving the combined protocol of hypothermic and normothermic MP showed that it potentially improves the reconditioning of ECD livers. However, whether all the potential advantageous effects seen for donor organs during the experiments are going to translate into clinical benefits to patients still need to be investigated.

The first clinical series using an end-ischaemic combined protocol of hypothermic and normothermic MP was recently published (2). This pilot study demonstrated clinically the safety and feasibility of this combined protocol using a single haemoglobin-based oxygen carrier (HBOC)-based perfusate throughout the entire perfusion. The 3-month survival rate was 100% for the five transplants performed (2). However, the absence of a control group limits further assessment of the clinical benefits of the combined protocol over isolated MP techniques. In addition, the assessment of post-transplant biliary complications was compromised by the non-realisation of imaging exams.

Therefore, the results from the two preclinical studies included in this thesis allied to those from the first clinical series demonstrating the safety and feasibility of a combined protocol of hypothermic and normothermic MP suggest that this newly developed protocol is a promising

future approach to increase ECD liver utilisation. Nevertheless, future randomised controlled trials using biliary complications as a primary endpoint and comparing the combined protocol with each technique independently are required to prove its superiority over isolated protocols.

### **9.2.2 Determining the safety of advancing ECD liver reconditioning during MP one step further**

The suggested improved reconditioning of ECD livers in an end-ischaemic combined protocol of MP may push the limits one step further for utilisation of donor organs with either unfavourable features (advanced age, steatosis, poor *in situ* flush) or damaged by prolonged periods of ischaemia, that would otherwise potentially fail to achieve transplantability criteria during normothermic machine perfusion (NMP). An interesting question is to determine whether there will be limits for utilisation of these ‘reconditioned’ organs. For example, future studies need to show the safety of transplanting ECD livers damaged by periods of ischaemia exceeding current acceptable standards prior to initiation of MP. Concisely, the benefits of transplanting these organs should exceed the associated risks, such as post-transplant biliary complications and graft dysfunction. To date, even though donation after circulatory death donor livers with prolonged warm ischaemia time were included in clinical studies, cold ischaemia time has been preserved within relatively acceptable limits prior to initiation of MP. In conclusion, the development of therapeutic interventions during MP, pharmacological or not, aiming to improve the reconditioning of the highest risk ECD livers is a new proposition demanding careful consideration to guarantee patient safety.

### **9.2.3 The utilisation of HBOC as an oxygen carrier replacing red blood cells in the perfusate for *ex situ* machine perfusion of donor livers**

Successful tissue oxygenation during NMP is based on the deployment of a perfusion fluid containing some sort of oxygen carrier. The commonest oxygen carrier used in this scenario, so far, is red blood cells (RBC). Whilst, due to their nature, RBC are safe, widely used and effective, their utilisation for this purpose requires caution. This is because RBC are a precious and scarce resource whose availability depends on voluntary donation and they are irreplaceable in the treatment of some life-threatening conditions, such as acute anaemia caused by surgical bleeding or in the context of trauma. In addition, RBC are associated with an increased risk of adverse immune reactions and infections (2–4). Considering the combination of hypothermic and normothermic MP, an RBC-based perfusate may not be used at hypothermic temperatures because of the risk of sludging (5) and haemolysis due to lower deformability of erythrocyte membranes at low temperatures (2).

Alternative oxygen carriers to replace RBC have been extensively investigated; a thorough examination of these molecules is beyond the scope of this thesis and can be found elsewhere (6, 7). In the context of MP of the liver, the polymerised bovine HBOC Hemopure<sup>®</sup> (hemoglobin glutamer-250 [bovine]; HBOC-201, Hemoglobin<sup>®</sup> Oxygen Therapeutics LLC, Cambridge, USA) was used in previous experimental studies (3, 8). Fontes *et al.* (2015) reported for the first time the use of an HBOC-based perfusate for subnormothermic machine perfusion (SMP) in a porcine liver transplant model (8). Six livers submitted to SMP were compared with static cold storage (SCS) preserved organs. The authors reported that SMP-treated organs presented higher 5-day survival (100% vs. 30%), higher bile production and integrity of hepatocytes' mitochondria after reperfusion. Finally, the authors conclude that an

HBOC-based perfusate offered adequate tissue oxygenation during SMP without evidence of oxidative damage to the organs (8).

More recently, two other studies reported the feasibility of using an HBOC-based perfusate for NMP of human donor livers (3, 9). In Birmingham, our machine perfusion group first tested *in vitro* the cytotoxicity of Hemopure® towards primary human hepatocytes and then perfused five human donor livers using an HBOC-based perfusate. Compared with an RBC-based perfusate, HBOC perfused livers extracted more oxygen without any evidence of tissue damage (3). Those findings were confirmed later by Matton *et al.*, who reported even higher ATP synthesis, cumulative bile production and vascular flows in liver perfused with an HBOC-based perfusate (9).

In **Chapter 4**, a new study was designed to test the feasibility of using an HBOC-based perfusate uninterruptedly from the hypothermic until the normothermic phase of the combined protocol of MP. This approach initially provided logistical advantages, such as avoidance of interruption of the perfusion to change the perfusate, which may increase its clinical applicability. To explore fully the advantages of having a perfusion fluid that could be used at variable temperatures, the organs were slowly rewarmed employing the Controlled Oxygenated Rewarming (COR) technique (10). COR avoids subtle temperature changes to the organs and has been shown to improve mitochondrial function with superior post-transplant outcomes compared with SCS (11).

This study tested the safety and stability of an HBOC-based perfusate for hypothermic oxygenated machine perfusion (HMP) of the liver. Although the HBOC-based perfusate was shown to be as effective as an oxygen-carrier-free perfusate at hypothermia, it facilitates the combination of hypothermic and normothermic perfusions, the Cold-to-Warm MP protocol. A

similar protocol was tested recently in a clinical series of five transplants, as described above, and the authors concluded that the use of an HBOC-based perfusate for MP of donor livers is apparently a safe alternative for RBC as an oxygen carrier in this scenario (2).

Therefore, mounting evidence suggests that HBOC may be a safe option to replace RBC as an oxygen carrier in the perfusate for *ex situ* MP of donor livers providing effective tissue oxygenation under variable temperatures. It may prevent the concerns associated with the use of RBC described above, and also offer other advantages, such as a long shelf life at room temperature, without the need for specific storage conditions and increase consistency between batches.

Whilst deployment of an HBOC-based perfusate confers several advantages, some potential limitations deserve further investigation. Previous clinical studies reported minor increases in methaemoglobin (MetHb) levels after transfusion of Hemopure<sup>®</sup> in humans (12, 13). MetHb is an oxidized form of haemoglobin that cannot bind oxygen and has a reduced ability to release oxygen to tissues (14). Physiologically, the erythrocytes' enzyme methaemoglobin reductase reduces the MetHb formed, keeping levels lower than 2% (15). Clinically, levels under 15% are only associated with greyish skin without further complications (14, 15). In the context of MP, a previous report on a porcine model showed stable MetHb levels of around 2% during SMP (8). We have found similar stable levels during HMP and rewarming, nevertheless, during NMP MetHb levels increased steadily, reaching median levels of around 7% at the end of three hours of NMP. Despite this increase, there was no suggestion of any ischaemic injury to the livers at this point; however, the long-term behaviour of this upward MetHb trend deserves further investigation.

In addition, the visual aspect of the bile during MP varies accordingly with the use of an HBOC- or an RBC-based perfusate. Grossly, the bile formed during MP using an HBOC-based perfusate is darker and more fluid. Further investigations on bile composition may clarify the reason for this difference and suggest whether it may have any clinical significance.

### **9.3 The *ex situ* manipulation of the hepatic lipid metabolism during normothermic machine perfusion**

The studies presented in **Chapters 5** and **6** showed that pharmacological modulation of the hepatic lipid metabolism during NMP can promote defatting of whole human livers within six hours. More importantly than reducing the lipid content, the enhanced lipid metabolism improved the metabolic status of the organs, optimised their functional recovery, decreased vascular resistance and reduced the expression of markers of ischaemia-reperfusion injury (IRI). These findings are promising and may guarantee future clinical investigation, opening a window of opportunity to improve the reconditioning of steatotic donor organs. Moreover, equally importantly, this study exemplifies the possible potential advantages of delivering therapeutic interventions during NMP and together with other strategies under investigation, such as stem cells treatment and immunological modification of the organs, defatting may potentially help to increase ECD liver utilisation (16).

All the exciting findings described above must be interpreted in the face of the limitations of the study. In the United Kingdom, histological assessment of donor liver steatosis is not performed routinely, and livers are discarded solely based on a surgical macroscopic assessment of steatosis. As a consequence, livers presenting with various grades of steatosis, assessed at the histological level, were included in the study, nonetheless, they were equally distributed

between groups. Another factor to be taken into consideration is the scarcity and heterogeneity amongst discarded human livers, which does not facilitate a perfect matching between experimental groups and limits sample size. In addition, this preclinical study is not a transplant model, yet transplantability was assessed using the clinically tested viability criteria (1). In accordance, development in the future of animal transplant models may provide additional evidence of the continuity of the benefits of the treatment after transplantation.

#### **9.4 Future research questions—Effect of defatting therapies on donor liver functional recovery**

After this thorough stepwise investigation for the delivery of a cocktail of defatting agents to human donor livers undergoing NMP in **Chapters 5–7**, numerous research questions may arise for future studies.

##### **9.4.1 The practical applicability of the defatting protocol in clinical transplantation**

The pharmacological enhancement of intracellular lipid metabolism during NMP not only decreased the lipid content of human donor livers but it also improved the intracellular metabolic support to the organs. All the organs that received the treatment reached the previously published viability criteria from Birmingham and, therefore, would potentially be deemed transplantable (1). Treated organs would meet similarly the viability criteria published recently by the Cambridge group, which considers also other additional factors (perfusate transaminases, bile production and bile quality) (17). Thus, this first preclinical study forms part of the basis for a future pilot study to assess the defatting protocol in clinical transplantation.

However, some questions may deserve discussion prior to its clinical application. Whilst the cytotoxicity of the defatting cocktail to primary human cells of the liver was tested prior to the delivery to human donor livers during NMP, the effect of the individual components of the cocktail in isolation was not evaluated. Therefore, future studies may determine whether all the drugs are necessary, or a refined combination provides a similar effect. In addition, the beneficial effects of the treatment were achieved predominantly within the initial six hours of perfusion, thereafter they seem to be maintained. It was hypothesised that the drugs have been already metabolised by the liver at this time point, whereas measurement of the drug concentration would be required to confirm this hypothesis. This former observation raises the question as to whether an additional bolus of drugs should be given to enhance the benefits to the organs, or the aim is considered achieved and the organ allocated for transplantation. In either case, assessment of the concentration of the drugs in the cold flush after perfusion, immediately before transplantation, must inform us about the risk of delivering the drugs to recipients. A negligible concentration must reassure the safety of the procedure and possibly hasten its clinical application.

#### **9.4.2 The potential beneficial impact of the defatting cocktail on post-transplant biliary complications**

A remarkable finding of this study was the positive impact of the treatment on biliary function. Watson *et al.* (2018) described that bile-pH < 7.5 during NMP is suggestive of biliary tree necrosis and can increase the risk of ischaemic-type biliary lesions (17). Contrary to this, treated livers demonstrated bile-pH levels higher than 7.5 whilst the converse was seen in control livers. In addition, defatted livers also produced a higher volume of bile independently of the increased



cholesterol levels in the perfusate. Altogether, it is suggested that the treatment had a beneficial effect on the biliary system by itself, although further investigation is needed to understand the mechanisms involved. The protective role of defatting therapies mitigating IRI, as represented by the lower oxidative tissue damage and alleviated activation of the inflammatory response may be potentially correlated. Furthermore, an increase in the secretion of phospholipids and cholesterol in the bile may equilibrate the phospholipids/bile salts balance, preventing further injury to the biliary tree. Thus, these observations may guarantee future studies investigating the delivery of pharmacological agents during NMP, which may potentially prevent the development of ischaemic-type biliary lesions post-transplantation.

#### **9.4.3 Mitigation of IRI is likely to be a key factor to increase safely the utilisation of steatotic donor livers**

Mitigation of IRI seems to be a key factor to improve the reconditioning of steatotic livers. Recently, He *et al.* published the first case of ischaemia-free organ transplantation (IFOT) for a severely steatotic donor liver (18). The authors reported that the recipient did not have post-reperfusion syndrome, and the organ demonstrated minimal signs of hepatobiliary injury and reduced pro-inflammatory response (18). These findings suggest that IFOT might be a promising approach to increase ECD livers' utilisation and reduce postoperative complications, whereas it should be highlighted that this technique is limited to donation after brainstem death donors and might be logistically challenging in a multivisceral retrieval setting.

Kron *et al.* (2017) in a transplant rat model of severely steatotic livers submitted to 12 hours of SCS showed that short-term hypothermic oxygenated perfusion (HOPE) prior to implantation was able to diminish IRI, as assessed by oxidative injury, nuclear damage, Kupffer and

endothelial cell activation, without changing the steatosis grading of the organs (19). The same study reported results of six moderately steatotic human livers that had HOPE prior to transplantation suggesting achievement of similar benefits. However, some observations deserve attention, such as the perfusion period was variable (1.8–4.8 hours), the livers had short cold ischaemia time (median 4.1 hours) and macrovesicular steatosis rates differ slightly from controls (HOPE 20–40% vs. controls 25–60%). Interestingly, the prolonged perfusion interval in comparison with the standard 1–2 hours of HOPE may indicate that the authors considered it necessary to downregulate mitochondrial respiration and potentially replenish cellular energy stores prior to transplantation.

These studies, in combination with the findings from this thesis, suggest that strategies to mitigate IRI might possibly drive the setting of a new benchmark for steatotic liver preservation.

## 9.5 Conclusion

**Chapters 1 and 2** discussed the need to increase ECD organs' utilisation to address the liver transplant waiting list mortality; and how the use of these marginal organs impacts negatively on postoperative outcomes. The current clinical evidence supporting machine perfusion of the liver as an alternative to recondition these risky organs was reviewed, and the advantages and limitations of different modalities of machine perfusion were described. **Chapter 3** presented a newly developed protocol of *ex situ* end-ischaemic machine perfusion of the liver, which combines HOPE and NMP. This unified protocol derived benefits of the individual techniques and increased the rate of donor organs recovering adequate metabolic parameters to be deemed transplantable. **Chapter 4** showed that it is feasible to use an acellular HBOC-based perfusate from hypothermic until normothermic perfusion. The use of an HBOC-based perfusate

facilitates the logistics of combining these protocols in an uninterrupted fashion from cold to warm, the Cold-to-Warm MP. **Chapter 5** reviewed the problematic features of fatty livers in the context of liver transplantation and the possible role of MP techniques. The concept of steatosis reversal or defatting was introduced, and the pharmacodynamics of the drugs utilised so far were discussed. **Chapter 6** first tested the cytotoxicity *in vitro* of a combination of defatting agents on primary human hepatocytes, cholangiocytes and intrahepatic endothelial cells. It was shown that the drugs are safe for those cells, and they were effective in reducing the lipid content of fat-laden primary human hepatocytes via upregulation of fatty acids'  $\beta$ -oxidation. **Chapter 7** described the first study designed to deliver the defatting drugs to donor human livers undergoing NMP. It was shown that the pharmacological modulation of the hepatic lipid metabolism during normothermic perfusion can promote defatting of whole human livers within six hours. The enhancement in the lipid metabolism improved the metabolic support to other cellular processes, improving the rescue of appropriate functional parameters of the organs and mitigating IRI-associated tissue damage. Treated livers exhibited improved biliary function, as represented by higher production of good quality bile; whilst mechanistically this is most likely to be associated with the alleviated IRI, other hypotheses are reviewed in **Chapter 8**. **Chapter 9** discussed future directions for studies in the field and revised key points of the two proposals investigated to improve the reconditioning of high-risk ECD organs in this thesis: (1) The use of hypothermic oxygenated machine perfusion as a therapeutic intervention preceding NMP; and (2) the delivery of a pharmacological combination of drugs targeting the hepatic lipid metabolism during NMP to enhance the metabolic support to donor organs.

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